BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

PCT RLD INTELLECTUAL PROPERTY ORGANIZAT
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07K 14/00	A2	(11) International Publication Number: WO 98/06747
		(43) International Publication Date: 19 February 1998 (19.02.98)

(21) International Application Number: PCT/US97/13910

(22) International Filing Date: 7 August 1997 (07.08.97)

(30) Priority Data:
08/696,566 16 August 1996 (16.08.96) US

(71) Applicant: GENENTECH, INC. [US/US]; 1 DNA Way, South San Francisco, CA 94080-4990 (US).

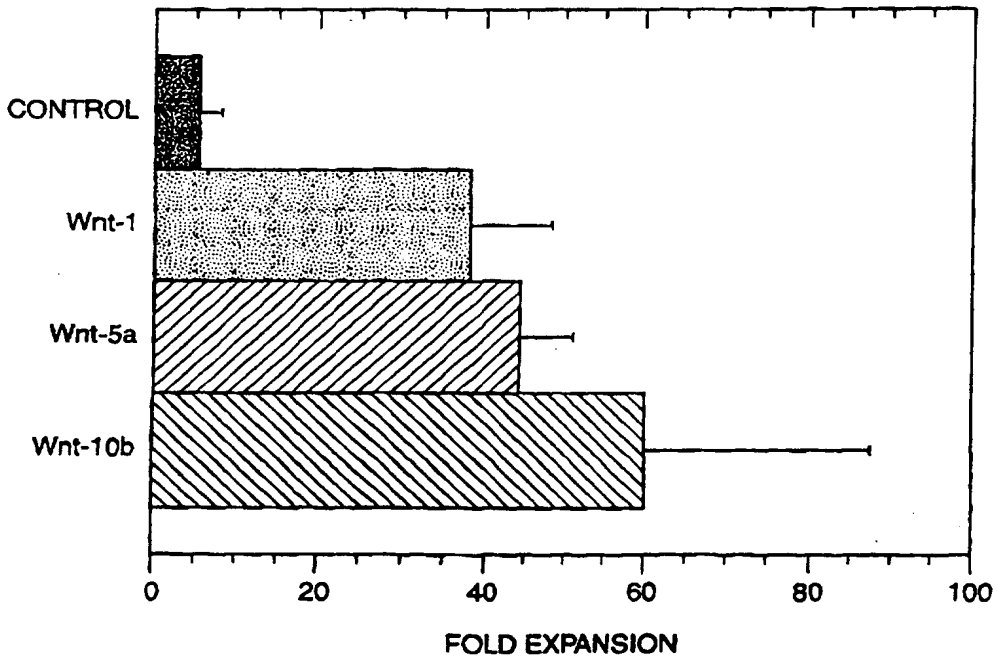
(72) Inventors: MATTHEWS, William; 560 Summit Springs Road, Woodside, CA 94062 (US). AUSTIN, Timothy, W.; 1010 Peebles Avenue, Morgan Hill, CA 95037 (US).

(74) Agents: SVOBODA, Craig, G. et al.; Genentech, Inc., 1 DNA Way, South San Francisco, CA 94080-4990 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: USES FOR WNT POLYPEPTIDES



(57) Abstract

Uses for *Wnt* polypeptides in hematopoiesis are disclosed. In particular, *in vitro* and *in vivo* methods for enhancing proliferation, differentiation or maintenance of a hematopoietic stem/progenitor cell using a *Wnt* polypeptide, and optionally another cytokine, are described.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

USES FOR WNT POLYPEPTIDES

FIELD OF THE INVENTION

This application relates to uses for Wnt polypeptides ("Wnts"). In particular, the invention relates to uses for a Wnt polypeptide for enhancing the proliferation, differentiation and/or maintenance of primitive hematopoietic cells, *e.g.*, hematopoietic stem/progenitor cells.

BACKGROUND OF THE INVENTION

A. HEMATOPOIESIS

The process of blood cell formation whereby red and white blood cells are replaced through the division of cells located in the bone marrow is called hematopoiesis. For a review of hematopoiesis see *Dexter and Spooncer* (Ann. Rev. Cell Biol., 3:423-441 [1987]).

There are many different types of blood cells which belong to distinct cell lineages. Along each lineage, there are cells at different stages of maturation. Mature blood cells are specialized for different functions. For example, erythrocytes are involved in O_2 and CO_2 transport; T and B lymphocytes are involved in cell and antibody mediated immune responses, respectively; platelets are required for blood clotting; and the granulocytes and macrophages act as general scavengers and accessory cells. Granulocytes can be further divided into basophils, eosinophils, neutrophils and mast cells.

Each of the various blood cell types arises from pluripotent or totipotent stem cells which are able to undergo self-renewal or give rise to progenitor cells or Colony Forming Units (CFU) that yield a more limited array of cell types. As stem cells progressively lose their ability to self-renew, they become increasingly lineage restricted. It has been shown that stem cells can develop into multipotent cells (called "CFC-Mix" by *Dexter and Spooncer, supra*). Some of the CFC-Mix cells can undergo renewal whereas others lead to lineage-restricted progenitors which eventually develop into mature myeloid cells (*e.g.*, neutrophils, megakaryocytes, macrophages, basophils and erythroid cells). Similarly, pluripotent stem cells are able to give rise to PreB and PreT lymphoid cell lineages which differentiate into mature B and T lymphocytes, respectively. Progenitors are defined by their progeny, *e.g.*, granulocyte/macrophage colony-forming progenitor cells (GM-CFU) differentiate into neutrophils or macrophages; primitive erythroid burst-forming units (BFU-E) differentiate into erythroid colony-forming units (CFU-E) which give rise to mature erythrocytes. Similarly, the Meg-CFU, Eos-CFU and Bas-CFU progenitors are able to differentiate into megakaryocytes, eosinophils and basophils, respectively.

The number of pluripotent stem cells in the bone marrow is extremely low and has been estimated to be in the order of about one per 10,000 to one per 100,000 cells (*Boggs et al.*, J. Clin. Inv., 70:242 [1982] and *Harrison et al.*, PNAS, 85; 822 [1988]). Accordingly, characterization of stem cells has been difficult. Therefore, various protocols for enriching pluripotent stem cells have been developed. See, for example, *Matthews et al.*, Cell, 65:1143-1152 [1991]; WO 94/02157; *Orlic et al.*, Blood, 82(3):762-770 [1993]; and *Visser et al.*, Stem Cells, 11(2):49-55 [1993].

Various lineage-specific factors have been demonstrated to control cell growth, differentiation and the functioning of hematopoietic cells. These factors or cytokines include the interleukins (*e.g.*, IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), granulocyte colony-stimulating factor (M-CSF), erythropoietin (Epo), lymphotoxin, steel factor (SLF), tumor necrosis factor (TNF) and gamma-interferon. These growth factors have a broad spectrum of activity, from generalized to lineage-specific roles in hematopoiesis, or a combination of both. For example, IL-3 appears to act on multipotent stem cells as well as progenitors restricted to the granulocyte/macrophage, eosinophil, megakaryocyte, erythroid or mast cell lineages. On the other hand, Epo generally acts on fairly mature erythroid progenitor cells.

B. THE HEMATOPOIETIC ENVIRONMENT AND EMBRYOGENESIS

The capacity of the hematopoietic stem cells to provide for the lifelong production of all blood lineages is accomplished by a balance between the plasticity of the stem cell, that is the production of committed progenitors cells which generate specific blood lineages, and the replication of the stem cell in the undifferentiated state (self-renewal). The mechanisms regulating hematopoietic stem cells' plasticity and self-renewal *in vivo* have been difficult to define. However, the major contributory factors represent a combination of cell intrinsic and environmental influences (*Morrison et al.*, Proc. Natl. Acad. Sci. USA, 92: 10302-10306 [1995]). The importance of the hematopoietic microenvironment has been established through the use of long term bone marrow culture systems where hematopoietic cells cultured on stroma allow for the maintenance of HSCs, albeit at low frequencies (*Fraser et al.*, Proc. Natl. Acad. Sci. USA, 89: 1968-1972, [1992]; *Wineman et al.*, Blood, 81: 365-372 [1993]).

The demonstration of hematopoietic cell maintenance in culture has led to efforts to identify candidate 'stem cell' factors. The role of hematopoietic cytokines in stem cell maintenance has been studied by direct addition of purified factors to *in vitro* cultures of stem cell populations followed by transplantation of the cultured cells (*Muench et al.*, Blood, 81: 3463-3473 [1993]; *Wineman et al.*, *supra* [1993]; *Rebel et al.*, Blood, 83: 128-136 [1994]). Most of the known 'early-acting' cytokines such as IL-3, IL-6, and KL have been shown to stimulate proliferation of more committed progenitor cells while concurrently allowing maintenance, but not expansion, of cells capable of long-term multilineage repopulation (reviewed in *Williams*, Blood, 12: 3169-3172 [1993]; *Müller-Sieberg and Deryugina*, Stem Cells, 13: 477-486 [1995]). While these data indicate that the cells' plasticity and repopulating function may be preserved by cytokine treatment, the molecules that promote self-renewal of these pluripotent cells remain unknown.

Transplantation studies have shown that the signals that regulate fate pluripotent stem cells may be similar in the embryo and adult bone marrow. Cells from the day 11 fetal liver, yolk sac, or aorta/gonad/mesonephros (AGM) region can repopulate the adult marrow and appropriately respond to extrinsic cues to sustain long-term multilineage hematopoiesis (*Müller et al.*, Immunity, 1: 291-301 [1994]). Although embryonic hematopoiesis is largely devoted to the erythroid lineage, the embryonic microenvironment clearly contributes to the maintenance of pluripotent stem cells in the undifferentiated state. These cell populations are

cycling during embryogenesis (*Zeigler et al., supra* [1994]; *Morrison et al., supra* [1995]; *Rebel et al.*, Blood, 87: 3500-3507 [1996]).

In mammals, hematopoietic precursors arise in the extraembryonic and ventral mesoderm, yolk sac, or AGM region (*Dzierzak and Medvinsky*, Trends Genet., 11: 359-366 [1995]; *Zon*, Blood, 86: 2876-2891 [1995]). In amphibian embryos, the equivalent regions are the ventral blood island mesoderm and the dorsal lateral plate mesoderm (reviewed in *Kessler and Melton*, Science, 266: 596-604 [1994]; *Zon, supra* 1995; *Tam and Quinlin*, Curr. Biol., 6: 104-106 [1996]). Secreted factors that potentially regulate cell fate determination of ventral mesoderm in *Xenopus* include *Wnts* , FGFs, and BMP-4 (reviewed in *Christian and Moon*, Bio Essays, 15: 135-140 [1993a]; *Zon, supra* [1995]). Embryonic expression of *XWnt*-8 (*Christian and Moon*, Genes Dev., 7: 13-28 [1993b]) and *XWnt*-11 (*Ku and Melton*, Development, 119: 1161-1173 [1993]) is localized to the area of prospective ventral and lateral mesoderm and *XWnt*-8 expression can be induced by ventralizing factors such as FGFs and BMP-4.

C. *THE WNTS GENE FAMILY*

Wnts are encoded by a large gene family whose members have been found in round worms, insects, cartilaginous fish and vertebrates (Sidow, 1994). Wnts are thought to function in a variety of developmental and physiological processes since many diverse species have multiple conserved *Wnt* genes (*McMahon*, Trends Genet., 8: 236-242 [1992]; *Nusse and Varmus*, Cell, 69: 1073-1087 [1992]). *Wnt* genes encode secreted glycoproteins that are thought to function as paracrine or autocrine signals active in several primitive cell types (*McMahon, supra* [1992]; *Nusse and Varmus, supra* [1992]). The Wnt growth factor family includes more than 10 genes identified in the mouse (*Wnt*-1, 2, 3a, 3b, 4, 5a, 5b, 6, 7a 7b, 8a, 8b, 10b, 11, 12) (see, *e.g.*, *Gavin et al.*, Genes Dev., 4: 2319-2332 [1990]; *Lee et al.*, Proc. Natl. Acad. Sci. USA, 92: 2268-2272; *Christiansen et al.*, Mech. Dev., 51: 341-350 [1995]) and at least 7 genes identified in the human (*Wnt*-1, 2, 3, 4, 5a, 7a and 7b) by cDNA cloning (see, *e.g.*, *Vant Veer et al.*, Mol.Cell.Biol., 4: 2532-2534 [1984]). The *Wnt-1* proto-oncogene (int-1) was originally identified from mammary tumors induced by mouse mammary tumor virus (MMTV) due to an insertion of viral DNA sequence (*Nusse and Varmus*, Cell, 31: 99-109 [1982]). In adult mice, the expression level of *Wnt*-1 mRNA is detected only in the testis during later stages of sperm development. Wnt-1 protein is about 42 KDa and contains an amino terminal hydrophobic region, which may function as a signal sequence for secretion (*Nusse and Varmus, supra*). The expression of *Wnt*-2/irp is detected in mouse fetal and adult tissues and its distribution does not overlap with the expression pattern for Wnt-1. *Wnt*-3 is associated with mouse mammary tumorigenesis. The expression of Wnt-3 in mouse embryos detected in the neural tubes and in the limb buds. *Wnt*-5a transcripts are detected in the developing fore- and hind limbs at 9.5 through 14.5 days and highest levels are concentrated in apical ectoderm at the distal tip of limbs (*Nusse and Varmus, supra* [1992]. Recently, a Wnt growth factor, termed Wnt-x, was described (PCT/US94/14708; WO95/17416) along with the detection of *Wnt*-x expression in bone tissues and in bone-derived cells. Also described was the role of Wnt-x in the maintenance of mature osteoblasts and the use of the Wnt-x growth factor as a therapeutic agent or in the development of other therapeutic agents to treat bone-related diseases.

Wnts may play a role in local cell signaling. Biochemical studies have shown that much of the secreted Wnt protein can be found associated with the cell surface or extracellular matrix rather than freely diffusible in

the medium (*Papkoff and Schryver*, Mol. Cell. Biol., 10: 2723-2730 [1990]; *Bradley and Brown*, EMBO J., 9: 1569-1575 [1990]).

Studies of mutations in *Wnt* genes have indicated a role for *Wnts* in growth control and tissue patterning. In *Drosophila*, *wingless* (*wg*) encodes a *Wnt* gene (*Rijsewik et al.*, Cell, 50: 649-657 [1987]) and *wg* mutations alter the pattern of embryonic ectoderm, neurogenesis, and imaginal disc outgrowth (*Morata and Lawerence*, Dev. Biol., 56: 227-240 [1977]; *Baker*, Dev. Biol., 125: 96-108 [1988]; *Klingensmith and Nusse*, Dev. Biol., 166: 396-414 [1994]). In *Caenorhabditis elegans*, *lin*-44 encodes a Wnt which is required for asymmetric cell divisions (*Herman and Horvitz*, Development, 120: 1035-1047 [1994]). Knock-out mutations in mice have shown Wnts to be essential for brain development (*McMahon and Bradley*, Cell, 62: 1073-1085 [1990]; *Thomas and Cappechi*, Nature, 346: 847-850 [1990]), and the outgrowth of embryonic primordia for kidney (*Stark et al.*, Nature, 372: 679-683 [1994]), tail bud (*Takada et al.*, Genes Dev., 8: 174-189 [1994]), and limb bud (*Parr and McMahon*, Nature, 374: 350-353 [1995]). Overexpression of *Wnts* in the mammary gland can result in mammary hyperplasia (*McMahon*, *supra* [1992]; *Nusse and Varmus*, *supra* [1992]), and precocious alveolar development (*Bradbury et al.*, Dev. Biol., 170: 553-563 [1995]). A role for *Wnts* in mammalian hematopoiesis has not previously been suggested or considered.

Wnt-5a and *Wnt*-5b are expressed in the posterior and lateral mesoderm and the extraembryonic mesoderm of the day 7-8 murine embryo (*Gavin et al.*, *supra* [1990]). These embryonic domains contribute to the AGM region and yolk sac tissues from which multipotent hematopoietic precursors and HSCs are derived (*Dzierzak and Medvinsky*, *supra* [1995]; *Zon*, *supra* [1995]; *Kanatsu and Nishikawa*, Development, 122: 823-830 [1996]). *Wnt*-5a, *Wnt*-10b, and other *Wnts* have been detected in limb buds, indicating possible roles in the development and patterning of the early bone microenvironment as shown for *Wnt*-7b (*Gavin et al.*, *supra* [1990]; *Christiansen et al.*, Mech. Devel., 51: 341-350 [1995]; *Parr and McMahon*, *supra* [1995]).

D. *HEMATOPOIETIC DISEASES AND DISORDERS*

Chemo- and radiation therapies cause dramatic reductions in blood cell populations in cancer patients. At least 500,000 cancer patients undergo chemotherapy and radiation therapy in the US and Europe each year and another 200,000 in Japan. Bone marrow transplantation therapy of value in aplastic anemia, primary immunodeficiency and acute leukemia (following total body irradiation) is becoming more widely practiced by the medical community. At least 15,000 Americans have bone marrow transplants each year. Other diseases can cause a reduction in entire or selected blood cell lineages. Examples of these conditions include anemia (including macrocytic and aplastic anemia); thrombocytopenia; hypoplasia; immune (autoimmune) thrombocytopenic purpura (ITP); and HIV induced ITP.

Pharmaceutical products are needed which are able to enhance reconstitution of blood cell populations of these patients.

Accordingly, it is an object of the present invention to provide a method for enhancing the proliferation and/or differentiation and/or maintenance of primitive hematopoietic cells. Such a method may be useful for enhancing repopulation of hematopoietic stem cells and thus mature blood cell lineages. This is desirable where a mammal has suffered a decrease in hematopoietic or mature blood cells as a consequence of disease, radiation

or chemotherapy. This method is also useful for generating expanded populations of such stem cells and mature blood cell lineages from such hematopoietic cells *ex vivo*.

These and other objects will be apparent to the ordinary artisan upon consideration of the specification as a whole.

SUMMARY OF THE INVENTION

In one aspect, the present invention pertains to the discovery herein that Wnt polypeptides ("Wnts"), such as Wnt-5a, play a role in hematopoiesis. In another aspect, the present invention is based on the observation that such Wnts function as hematopoietic regulatory factors and are able to directly stimulate the proliferation of hematopoietic stem cells, trigger the formation of multicellular aggregates or 'foci' of primitive blast cells, and expand the total number of multipotential colony forming cells via receptor signalling. Wnts appeared to directly act at the level of the early hematopoietic precursor (*i.e.*, hematopoietic stem/progenitor cells). Such an expanded stem cell population can serve as the source of cells for myelopoiesis, erythropoiesis (*e.g.*, splenic erythropoiesis) and lymphopoiesis. Accordingly, Wnts can be used to stimulate proliferation and/or differentiation and/or maintenance of hematopoietic stem/progenitor cells either *in vitro* or *in vivo* (*e.g.*, for treating hematopoietic diseases or disorders).

Thus, the invention provides a method for enhancing proliferation, differentiation and/or maintenance of a cell with a Wnt polypeptide comprising the step of contacting the cell with an amount of Wnt polypeptide which is effective for stimulating proliferation and/or differentiation and/or maintenance (*e.g.*, survival) of the cell. In preferred embodiments, the cell which is exposed to the Wnt polypeptide is a hematopoietic precursor, *e.g.*, a hematopoietic stem/progenitor cell. For example, the Wnt polypeptide may be Wnt-1, Wnt-5a or Wnt-10b. For *in vivo* use, the Wnt polypeptide of choice may be a long half-life derivative of, for example, a Wnt-5a polypeptide such as Wnt-5a immunoglobulin chimera and/or Wnt-5a polypeptide modified with a nonproteinaceous polymer, such as polyethylene glycol (PEG). The method contemplated herein may lead to an increase in the proliferation and/or differentiation of lymphoid, myeloid and/or erythroid blood cell lineages from the maintained or expanded hematopoietic stem/progenitor cell population and encompasses both *in vitro* and *in vivo* methods. For *in vitro* uses, the cell stimulated by Wnts may be present in cell culture. As to *in vivo* methods, the cell may be present in a mammal, especially a human (*e.g.*, one who is suffering from decreased blood levels and who could benefit from an increase in various blood cells). Potential patients include those who have undergone chemo- or radiation therapy, or bone marrow transplantation therapy. Thus, the invention provides a method for repopulating blood cells (*e.g.*, erythroid, myeloid and/or lymphoid blood cells) in a mammal comprising administering to the mammal a therapeutically effective amount of Wnt polypeptide.

Mammals which may benefit from an enhancement of lymphopoiesis include those predisposed to, or suffering from, any one or more of the following exemplary conditions: lymphocytopenia; lymphorrhea; lymphostasis; immunodeficiency (*e.g.*, HIV and AIDS); infections (including, for example, opportunistic infections and tuberculosis (TB)); lupus; and other disorders characterized by lymphocyte deficiency. An effective amount of the Wnt polypeptide can be used in a method of immunopotentiation or to improve immune function in a mammal.

Diseases or disorders in which an increase in erythropoiesis may be beneficial include, but are not limited to: erythrocytopenia; erthrodegenerative disorders; erythroblastopenia; leukoerythroblastosis; erythroclasis; thalassemia; and anemia (*e.g.*, hemolytic anemia, such as acquired, autoimmune, or microangiopathic hemolytic anemia; aplastic anemia; congenital anemia, *e.g.*, congenital dyserythropoietic anemia, congenital hemolytic anemia or congenital hypoplastic anemia; dyshemopoietic anemia; Faconi's anemia; genetic anemia; hemorrhagic anemia; hyperchromic or hypochromic anemia; nutritional, hypoferric, or iron deficiency anemia; hypoplastic anemia; infectious anemia; lead anemia; local anemia; macrocytic or microcytic anemia; malignant or pernicious anemia; megaloblastic anemia; molecular anemia; normocytic anemia; physiologic anemia; traumatic or posthemorrhagic anemia; refractory anemia; radiation anemia; sickle cell anemia; splenic anemia; and toxic anemia).

An increase in myelopoiesis may be beneficial in any of the above-mentioned diseases or disorders as well as the following exemplary conditions; myelofibrosis; thrombocytopenia; hypoplasia; disseminated intravascular coagulation (DIC); immune (autoimmune) thrombocytopenic purpura (ITP); HIV inducted ITP; myelodysplasia; thrombocytotic diseases and thrombocytosis.

The method may further involve the step of exposing hematopoietic cells (whether they be in cell culture or in a mammal) to one or more other cytokines (*e.g.*, lineage-specific cytokines) and this may lead to a synergistic enhancement of the proliferation and/or differentiation of the cells. Exemplary cytokines include thrombopoietin (TPO); erythropoietin (EPO); macrophage-colony stimulating factor (M-CSF); granulocyte-macrophage-CSF (GM-CSF); granulocyte-CSF (G-CSF); interleukin-1 (IL-1); IL-1a; IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9; IL-11; IL10; IL-12; leukemia inhibitory factor (LIF) or kit ligand (KL). In this embodiment, exposure to the cytokine may proceed, occur simultaneously with, or follow, exposure to the Wnt polypeptide. Preferably, the Wnt polypeptide and one or more further cytokines are administered simultaneously to the patient (where the method is an *in vivo* one) and, optionally, are combined to form a pharmaceutical composition.

For use in the above methods, the invention also provides an article of manufacture, comprising: a container, a label on the container, and a composition comprising an active agent within the container, wherein the composition is effective for enhancing proliferation and/or differentiation and/or maintenance of hematopoietic stem/progenitor cells in a mammal, the label on the container indicates that the composition can be used for enhancing proliferation and/or differentiation and/or maintenance of those cells and the active agent in the composition is a Wnt polypeptide. Optionally, the article of manufacture includes one or more further containers which hold further cytokine(s) in a packaged combination with the container holding the Wnt polypeptide.

In another embodiment, an effective amount of the Wnt polypeptide may be used to improve engraftment in bone marrow transplantation or to stimulate mobilization and/or expansion of hematopoietic stem cells in a mammal prior to harvesting hematopoietic progenitors from the peripheral blood thereof.

In addition to the above, the invention provides isolated nucleic acid molecules, expression vectors and host cells encoding a Wnt polypeptide which can be used in the recombinant production of Wnts as described herein. The isolated nucleic acid molecules and vectors are also useful for gene therapy applications to treat

patients, for example, to increase the number of cells expressing a Wnt polypeptide and increase Wnt responsiveness. In addition, anti-Wnt antibodies, in particular, neutralizing antibodies to Wnts, are useful for the treatment of disorders, stem cell tumors and other tumors at sites of Wnt expression, including those tumors characterized by overexpression of Wnts.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph showing Wnts promotion of cell proliferation in suspension cultures of flASK cells. The fold expansions in cell number following culture for 7 days are shown. Cultures were initiated with flASK cells (5000/well), 25 ng/ml KL, and conditioned media (CM) from 293 cells transfected with control plasmid, *Wnt*-1, *Wnt*-5a (gDWnt5aHis$_6$), or *Wnt*-10b. Assays were performed in duplicate and repeated in two independent experiments.

Figure 2 A, B and C are graphs showing Wnts promotion of enhanced fold expansion and colony formation from flASK cells. Figure 2A shows enhanced survival/proliferation of flASK cells following transduction with the Wnt5a/LNL6 retrovirus. Transductions were initiated with 100,000 cells/ml in IL-3, IL-6, and KL. The fold expansion for LNL6 or Wnt5a/LNL6-treated cells was determined from cell counts at the end of the transduction period (48 hours) and repeated four times. Figure 2B shows that suspension culture of Wnt5a/LNL6 transduced cells for 7 days results in extensive expansion compared to LNL6-treated cultures. Figure 2C shows the colony formation from flASK cells following a 48 hour transduction with LNL6 or Wnt5a/LNL6. Cells were plated in quadruplicate in myeloid methylcellulose, colony growth was evaluated after day 12 of culture, and repeated in four independent experiments.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

A search to uncover novel self-renewal factors from investigations on the embryonic and fetal hematopoietic microenvironment has led to the discovery that Wnt polypeptides comprise a novel class of stem cell regulators and directly stimulate the extensive proliferation and/or differentiation and/or maintenance of cultured hematopoietic stem/progenitor cells. Wnt polypeptides are thus useful *in vivo* or *ex vivo* to enhance proliferation and/or differentiation and/or maintenance of hematopoietic stem/progenitor cells, expand the population of these cells and enhance repopulation of such cells and blood cells of multiple lineages in a mammal.

I. Definitions

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

The terms "Wnts" or "Wnt gene product" or "Wnt polypeptide" when used herein encompass native sequence Wnt polypeptides, Wnt polypeptide variants, Wnt polypeptide fragments and chimeric Wnt polypeptides. Optionally, the Wnt polypeptide is not associated with native glycosylation. "Native glycosylation" refers to the carbohydrate moieties which are covalently attached to Wnt polypeptide when it is produced in the mammalian cell from which it is derived in nature. Accordingly, a human Wnt polypeptide produced in a non-human cell is an example of a Wnts which is "not associated with native glycosylation".

Sometimes, the Wnt polypeptide is unglycosylated (*e.g.*, as a result of being produced recombinantly in a prokaryote).

A "native sequence" polypeptide is one which has the same amino acid sequence as a polypeptide (*e.g.*, Wnt polypeptide) derived from nature. Such native sequence polypeptides can be isolated from nature or can be produced by recombinant or synthetic means. Thus, a native sequence polypeptide can have the amino acid sequence of naturally occurring human polypeptide, murine polypeptide, or polypeptide from any other mammalian species.

The term "native sequence Wnt polypeptide" includes those Wnt polypeptides from any animal species (*e.g.*, human, murine, rabbit, cat, cow, sheep, chicken, procine, equine, etc.) as occurring in nature. The definition specifically includes human Wnt polypeptides, Wnt-1, 2, 3, 4, 5a, 7a and 7b and murine Wnt polypeptides, Wnt-1, 2, 3a, 3b, 4, 5a, 5b, 6, 7a, 7b, 8a, 8b, 10b, 11 and 12. The term "native sequence Wnt protein" includes the native proteins with or without the initiating N-terminal methionine (Met), and with or without the native signal sequence. The native sequence human and murine Wnt polypeptides known in the art are from about 348 to about 389 amino acids long in their unprocessed form reflecting variability (particularly at the poorly conserved amino-terminus and several internal sites), contain 21 conserved cysteines, and have the features of a secreted protein (see, *e.g.*, Wnt polypeptides as in *Gavin et al., supra*; *Lee et al., supra*; *Christiansen et al., supra*; PCT/US94/140708 [WO 95/17416]). The molecular weight of a Wnt polypeptide is about 38-42 kD in a monomeric form.

A "variant" polypeptide means a biologically active polypeptide as defined below having less than 100% sequence identity with a native sequence polypeptide. Such variants include polypeptides wherein one or more amino acid residues are added at the N- or C-terminus of, or within, the native sequence; from about one to forty amino acid residues are deleted, and optionally substituted by one or more amino acid residues; and derivatives of the above polypeptides, wherein an amino acid residue has been covalently modified so that the resulting product has a non-naturally occurring amino acid. Ordinarily, a biologically active Wnt variant will have an amino acid sequence having at least about 90% amino acid sequence identity with a native sequence Wnt polypeptide, preferably at least about 95%, more preferably at least about 99%.

A "chimeric" Wnt polypeptide is a polypeptide comprising a Wnt polypeptide or portion (*e.g.*, one or more domains) thereof fused or bonded to heterologous polypeptide. The chimeric Wnt polypeptide will generally share at least one biological property in common with a native sequence Wnt polypeptide, such as Wnt-5a. Examples of chimeric polypeptides include immunoadhesins and epitope tagged polypeptides.

The term "Wnt immunoadhesin" is used interchangeably with the expression "Wnt polypeptide-immunoglobulin chimera" and refers to a chimeric molecule that combines a portion of the Wnt polypeptide with an immunoglobulin sequence. The immunoglobulin sequence preferably, but not necessarily, is an immunoglobulin constant domain. The immunoglobulin moiety in the chimeras of the present invention may be obtained from IgG1, IgG2, IgG3 or IgG4 subtypes, IgA, IgE, IgD or IgM, but preferably IgG1 or IgG3.

The term "epitope tagged" when used herein refers to a chimeric polypeptide comprising a Wnt polypeptide or portion thereof fused to a "tag polypeptide". The tag polypeptide has enough residues to provide an epitope against which an antibody thereagainst can be made, yet is short enough such that it does not interfere

with biological activity of the Wnt polypeptide. The tag polypeptide preferably also is fairly unique so that the antibody thereagainst does not substantially cross-react with other epitopes. Suitable tag polypeptides generally have at least six amino acid residues and usually between about 6-60 amino acid residues.

"Isolated" Wnt polypeptide means has been purified from a Wnts source or has been prepared by recombinant or synthetic methods and is sufficiently free of other peptides or proteins (1) to obtain at least 15 and preferably 20 amino acid residues of the N-terminal or of an internal amino acid sequence by using a spinning cup sequenator or the best commercially available amino acid sequenator marketed or as modified by published methods as of the filing date of this application, or (2) to homogeneity by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Homogeneity here means less than about 5% contamination with other source proteins.

"Essentially pure" protein means a composition comprising at least about 90% by weight of the protein, based on total weight of the composition, preferably at least about 95% by weight. "Essentially homogeneous" protein means a composition comprising at least about 99% by weight of protein, based on total weight of the composition.

"Biological property" when used in conjunction with either "Wnt polypeptide" or "isolated Wnt polypeptide" means having an effector function that is directly or indirectly caused or performed by native sequence Wnt polypeptide, such as Wnt-5a. Effector functions of native sequence Wnt polypeptides include enhancement of differentiation and/or proliferation and/or maintenance of hematopoietic/progenitor cells (*e.g.*, as determined in assays described in Examples 1 and 2). A "biologically active Wnt polypeptide" is one which possesses a biological property of native sequence Wnt polypeptide.

A "functional derivative" of a native sequence Wnt polypeptide is a compound having a qualitative biological property in common with a native sequence Wnt polypeptide. "Functional derivatives" include, but are not limited to, fragments of a native sequence and derivatives of a native sequence Wnt polypeptide and its fragments, provided that they have a biological activity in common with a corresponding native sequence Wnt polypeptide. The term "derivative" encompasses both amino acid sequence variants of Wnt polypeptide and covalent modifications thereof.

The phrase "long half-life" as used in connection with Wnt polypeptides, concerns Wnt derivatives having a longer plasma half-life and/or slower clearance than a corresponding native sequence Wnt polypeptide. The long half-life derivatives preferably will have a half-life at least about 1.5-times longer than a native Wnt polypeptide; more preferably at least about 2-times longer than a native Wnt polypeptide, more preferably at least about 3-times longer than a native Wnt polypeptide.

"Percent amino acid sequence identity" is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the residues in the native sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. None of N-terminal, C-terminal, or internal extensions, deletions, or insertions into the candidate sequence shall be construed as affecting sequence identity or homology.

The expression "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, a ribosome binding site, and possibly, other as yet poorly understood sequences. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adapters or linkers are used in accordance with conventional practice.

As used herein, the expressions "cell," "cell line," and "cell culture" are used interchangeably and all such designations include progeny. Thus, the words "transformants" and "transformed cells" or "transfectants" and "transfected cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies, antibody compositions with polyepitopic specificity, bispecific antibodies, diabodies, and single-chain molecules, as well as antibody fragments (*e.g.*, Fab, $F(ab)_2$, and Fv), so long as they exhibit the desired biological activity.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by *Kohler et al.*, Nature 256:495 [1975], or may be made by recombinant DNA methods (see, *e.g.*, U.S. Patent No. 4,816,567 (*Cabilly et al.*)). The "monoclonal antibodies" may also be isolated from

phage antibody libraries using the techniques in *Clackson et al.*, Nature 352:624-628 [1991] and *Marks et al.*, J. Mol. Biol. 222:581-597 [1991], for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (*Cabilly et al., supra*; *Morrison et al.*, Proc.Natl.Acad.Sci.USA, 81:6851-6855 [1984]).

"Humanized" forms of non-human (*e.g.*, murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary-determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see *Jones et al.*, Nature 321:522-525 [1986]; *Reichmann et al.*, Nature 332:323-329 [1988]; and *Presta*, Curr.Op.Struct.Biol. 2:593-596 [1992]. The humanized antibody includes a Primatized® antibody wherein the antigen-binding region of the antibody is derived from an antibody produced by immunizing macaque monkeys with the antigen of interest.

"Non-immunogenic in a human" means that upon contacting the polypeptide of interest in a physiologically acceptable carrier and in a therapeutically effective amount with the appropriate tissue of a human, no state of sensitivity or resistance to the polypeptide of interest is demonstrated upon the second administration of the polypeptide of interest after an appropriate latent period (*e.g.*, 8 to 14 days).

The phrase "enhancing proliferation of a cell" encompasses the step of increasing the extent of growth and/or reproduction of the cell relative to an untreated cell either *in vitro* or *in vivo*. An increase in cell proliferation in cell culture can be detected by counting the number of cells before and after exposure to a molecule of interest. The extent of proliferation can be quantified via microscopic examination of the degree of confluency. Cell proliferation can also be quantified using a thymidine incorporation assay.

By "enhancing differentiation of a cell" is meant the act of increasing the extent of the acquisition or possession of one or more characteristics or functions which differ from that of the original cell (*i.e.*, cell *specialization*). This can be detected by screening for a change in the phenotype of the cell (*e.g.*, identifying morphological changes in the cell and/or surface markers on the cell).

By "enhancing survival or maintenance of a cell" encompasses the step of increasing the extent of the possession of one or more characteristics or functions which are the same as that of the original cell (*i.e.*, cell phenotype maintenance). This can be detected by screening for the maintenance of the cell's phenotype (*e.g.*, blast cell phenotype as in Example 2).

A "hematopoietic stem/progenitor cell" or "primitive hematopoietic cell" is one which is able to differentiate to form a more committed or mature blood cell type.

A "hematopoietic stem cell" or "stem cell" is one which is specifically capable of long-term engraftment of a lethally irradiated host.

"Lymphoid blood cell lineages" are those hematopoietic precursor cells which are able to differentiate to form lymphocytes (B-cells or T-cells). Likewise, "lymphopoeisis" is the formation of lymphocytes.

"Erythroid blood cell lineages" are those hematopoietic precursor cells which are able to differentiate to form erythrocytes (red blood cells) and "erythropoeisis" is the formation of erythrocytes.

The phrase "myeloid blood cell lineages", for the purposes herein, encompasses all hematopoietic precursor cells, other than lymphoid and erythroid blood cell lineages as defined above, and "myelopoiesis" involves the formation of blood cells (other than lymphocytes and erythrocytes).

A "$CD34^+$ cell population" is enriched for hematopoietic stem cells. A $CD34^+$ cell population can be obtained from umbilical cord blood or bone marrow, for example. Human umbilical cord blood $CD34^+$ cells can be selected for using immunomagnetic beads sold by Miltenyi (California), following the manufacturer's directions.

An "$AA4^+$ cell population" is enriched for hematopoietic stem cells. An $AA4^+$ cell population can be obtained from fetal liver for example. $AA4^+$ cells can be selected by immunoadherent panning, for example, with an antibody such as AA4.1.

A "$Lin^{lo}Sca^+$", "cell population" or "$AA4^+Sca^+$ cell population" is enriched for hematopoietic stem cells. Such populations can be obtained from bone marrow or fetal liver, respectfully, for example. $Lin^{lo}Sca^+$ cells or $AA4^+Sca^+$ cells can be selected by cell sorting after staining with an antibody to the Sca-1 antigen (Ly6A/E), for example, the Ly6A/E phycoerythrin conjugate from Pharmingen (San Diego, CA).

A "flASK cell population" is highly enriched for hematopoietic stem cells from fetal liver. A flASK cell is a fetal liver, $AA4^+$, Sca^+, kit^+ cell. flASK cells can be selected by cell sorting after staining, for example, with antibodies.

"Physiologically acceptable" carriers, excipients, or stabilizers are ones which are nontoxic to the cell or mammal being exposed thereto at the dosages and concentrations employed. Often the physiologically acceptable carrier is an aqueous pH buffered solution. Examples of physiologically acceptable carriers include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronics or polyethylene glycol (PEG).

The term "cytokine" is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, growth factors and traditional polypeptide hormones. Included among the cytokines are growth hormones such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen, OB protein; tumor necrosis factor-α and -β; mullerian-inhibitingsubstance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF-β; platelet-growth factor; transforming growth factors (TGFs) such as TGF-α and TGF-β; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon-α, -β and -γ; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF(GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12; and other polypeptide factors including leukemia inhibitory factor (LIF) and kit ligand (KL). As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture and biologically active equivalents of the native sequence cytokines.

A "lineage-specific cytokine" is one which acts on relatively committed cells in the hematopoietic cascade and gives rise to an expansion in blood cells of a single lineage. Examples of such cytokines include EPO, TPO, and G-CSF.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disease or disorder as well as those in which the disease or disorder is to be prevented.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

By "solid phase" is meant a non-aqueous matrix to which a reagent of interest (*e.g.*, the Wnt polypeptide or an antibody thereto) can adhere. Examples of solid phases encompassed herein include those formed partially or entirely of glass (*e.g.*, controlled pore glass), polysaccharides (*e.g.*, agarose), polyacrylamides, polystyrene, polyvinyl alcohol and silicones. In certain embodiments, depending on the context, the solid phase can comprise the well of an assay plate; in others it is a purification column (*e.g.*, an affinity chromatography column). This term also includes a discontinuous solid phase of discrete particles, such as those described in U.S. Patent No. 4,275,149.

II. Modes for Carrying Out the Invention

The present invention is based on the discovery of the uses of Wnt polypeptides to enhance hematopoiesis. The experiments described herein demonstrate that Wnts function as hematopoietic regulatory factors which appear to play a role in enhancing proliferation, differentiation and/or maintenance of *hematopoietic* cells. In particular, Wnts have been found to be present in enriched human stem cell populations, and Wnts may be used to stimulate proliferation of hematopoietic stem cells/progenitor cells. Other uses for

these polypeptides will be apparent from the following discussion. A description follows as to how Wnt genes and polypeptides may be prepared.

A. Preparation of *Wnt* Genes and Gene Products

Most of the discussion below pertains to recombinant production of *Wnt* genes and gene products by culturing cells transformed with a vector containing Wnt polypeptide-encoding nucleic acid and recovering the polypeptide from the cell culture.

1. Isolation of DNA Encoding Wnt Polypeptide

The DNA encoding Wnt polypeptide may be obtained from any cDNA library prepared from tissue believed to possess the Wnt polypeptide mRNA and to express it at a detectable level. Accordingly, Wnt polypeptide DNA can be conveniently obtained from a cDNA library prepared from mammalian fetal liver or fetal brain. The Wnt polypeptide-encoding gene may also be obtained from a genomic library or by oligonucleotide synthesis.

Libraries are screened with probes (such as antibodies to the Wnt polypeptide, or oligonucleotides of about 20-80 bases) designed to identify the gene of interest or the protein encoded by it. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures as described in chapters 10-12 of *Sambrook et al., Molecular Cloning: A Laboratory Manual* (New York: Cold Spring Harbor Laboratory Press, 1989). An alternative means to isolate the gene encoding Wnt polypeptide is to use PCR methodology as described in section 14 of *Sambrook et al., supra.*

A preferred method of practicing this invention is to use carefully selected oligonucleotide sequences to screen cDNA libraries from various human tissues, preferably human fetal liver. The oligonucleotide sequences selected as probes should be of sufficient length and sufficiently unambiguous that false positives are minimized.

The oligonucleotide must be labeled such that it can be detected upon hybridization to DNA in the library being screened. The preferred method of labeling is to use ^{32}P-labeled ATP with polynucleotide kinase, as is well known in the art, to radiolabel the oligonucleotide. However, other methods may be used to label the oligonucleotide, including, but not limited to, biotinylation or enzyme labeling.

Amino acid sequence variants of Wnt polypeptide are prepared by introducing appropriate nucleotide changes into the Wnt polypeptide DNA, or by synthesis of the desired Wnt polypeptide. Such variants represent insertions, substitutions, and/or specified deletions of, residues within or at one or both of the ends of the amino acid sequence of a naturally occurring human Wnt polypeptide. Preferably, these variants represent insertions and/or substitutions within or at one or both ends of the mature sequence, and/or insertions, substitutions and/or specified deletions within or at one or both of the ends of the signal sequence of the Wnt polypeptide. Any combination of insertion, substitution, and/or specified deletion is made to arrive at the final construct, provided that the final construct possesses the desired biological activity as defined herein. The amino acid changes also may alter post-translational processes of the Wnt polypeptide, such as changing the number or position of glycosylation sites, altering the membrane anchoring characteristics, and/or altering the intracellular location of the Wnt polypeptide by inserting, deleting, or otherwise affecting the leader sequence of the Wnt polypeptide.

Variations in the native sequence as described above can be made using any of the techniques and guidelines for conservative and non-conservative mutations set forth in U.S. Pat. No. 5,364,934. These include oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR mutagenesis. See also, for example, Table I therein and the discussion surrounding this table for guidance on selecting amino acids to change, add, or delete.

2. Insertion of Nucleic Acid into Replicable Vector

The nucleic acid (*e.g.*, cDNA or genomic DNA) encoding the Wnt polypeptide is inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Many vectors are available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

a. *Signal sequence component*

The Wnt polypeptide useful in hematopoiesis according to the invention may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which is preferably a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the Wnt polypeptide DNA that is inserted into the vector. The heterologous signal sequence selected preferably is one that is recognized and processed (*i.e.*, cleaved by a signal peptidase) by the host cell. For prokaryotic host cells that do not recognize and process the native Wnt polypeptide signal sequence, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the native signal sequence may be substituted by, *e.g.*, the yeast invertase leader, α factor leader (including *Saccharomyces* and *Kluyveromyces* α-factor leaders, the latter described in U.S. Pat. No. 5,010,182 issued 23 April 1991), or acid phosphatase leader, the *C. albicans* glucoamylase leader (EP 362,179 published 4 April 1990), or the signal described in WO 90/13646 published 15 November 1990. In mammalian cell expression the native signal sequence (*e.g.*, the Wnt polypeptide presequence that normally directs secretion of Wnt polypeptide from human cells *in vivo*) is satisfactory, although other mammalian signal sequences may be suitable, such as signal sequences from other animal Wnt polypeptide, and signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders, for example, the herpes simplex gD signal.

The DNA for such precursor region is ligated in reading frame to DNA encoding the mature Wnt polypeptide.

b. *Origin of replication component*

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2μ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning

vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

Most expression vectors are "shuttle" vectors, *i.e.*, they are capable of replication in at least one class of organisms but can be transfected into another organism for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

DNA may also be amplified by insertion into the host genome. This is readily accomplished using *Bacillus* species as hosts, for example, by including in the vector a DNA sequence that is complementary to a sequence found in *Bacillus* genomic DNA. Transfection of *Bacillus* with this vector results in homologous recombination with the genome and insertion of Wnt polypeptide DNA. However, the recovery of genomic DNA encoding Wnt polypeptide is more complex than that of an exogenously replicated vector because restriction enzyme digestion is required to excise the Wnt polypeptide DNA.

c. ***Selection gene component***

Expression and cloning vectors should contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, *e.g.*, ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, *e.g.*, the gene encoding D-alanine racemase for *Bacilli*.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin, mycophenolic acid and hygromycin.

Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the Wnt polypeptide nucleic acid, such as DHFR or thymidine kinase. The mammalian cell transformants are placed under selection pressure that only the transformants are uniquely adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the transformants under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes Wnt polypeptide. Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Increased quantities of Wnt polypeptide are synthesized from the amplified DNA. Other examples of amplifiable genes include metallothionein-I and -II, preferably primate metallothionein genes, adenosine deaminase, ornithine decarboxylase, *etc.*

For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by *Urlaub et al.*, Proc. Natl. Acad. Sci. USA, 77: 4216

[1980]. The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies of the DHFR gene, and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding Wnt polypeptide. This amplification technique can be used with any otherwise suitable host, *e.g.*, ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant DHFR gene that is highly resistant to Mtx is employed (EP 117,060).

Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding Wnt polypeptide, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3'-phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, *e.g.*, kanamycin, neomycin, or G418. See U.S. Patent No. 4,965,199.

A suitable selection gene for use in yeast is the *trp*1 gene present in the yeast plasmid YRp7 (*Stinchcomb et al.*, Nature, 282: 39 [1979]). The *trp*1 gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1. *Jones*, Genetics, 85:12 (1977). The presence of the *trp*1 lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, *Leu*2-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the *Leu*2 gene.

In addition, vectors derived from the 1.6 μm circular plasmid pKD1 can be used for transformation of *Kluyveromyces* yeasts. *Bianchi et al.*, Curr. Genet., 12: 185 [1987]. More recently, an expression system for large-scale production of recombinant calf chymosin was reported for *K. lactis*. *Van den Berg*, Bio/Technology, 8: 135 [1990]. Stable multi-copy expression vectors for secretion of mature recombinant human serum albumin by industrial strains of *Kluyveromyces* have also been disclosed. *Fleer et al.*, Bio/Technology, 9: 968-975 [1991].

d. *Promoter Component*

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the Wnt polypeptide nucleic acid. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of particular nucleic acid sequence, such as the Wnt polypeptide nucleic acid sequence, to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, *e.g.*, the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to Wnt polypeptide-encoding DNA by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native Wnt polypeptide promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the Wnt polypeptide DNA. However, heterologous promoters are preferred, as they generally permit greater transcription and higher yields of Wnt polypeptide as compared to the native Wnt polypeptide promoter.

Promoters suitable for use with prokaryotic hosts include the β-lactamase and lactose promoter systems (*Chang et al.*, Nature, 275:615 [1978]; *Goeddel et al.*, Nature, 281: 544 [1979]), alkaline phosphatase, a tryptophan (trp) promoter system (*Goeddel*, Nucleic Acids Res., 8: 4057 [1980]; EP 36,776), and hybrid promoters such as the tac promoter. *deBoer et al.*, Proc. Natl. Acad. Sci. USA, 80: 21-25 [1983]. However, other known bacterial promoters are suitable. Their nucleotide sequences have been published, thereby enabling a skilled worker operably to ligate them to DNA encoding Wnt polypeptide (*Siebenlist et al.*, Cell, 20: 269 [1980]) using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding Wnt polypeptide.

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglyceratekinase (*Hitzeman et al.*, J. Biol. Chem., 255: 2073 [1980]) or other glycolytic enzymes (*Hess et al.*, J. Adv. Enzyme Reg., 7: 149 [1968]; *Holland*, Biochemistry, 17: 4900 [1978]), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657. Yeast enhancers also are advantageously used with yeast promoters.

Wnt polypeptide transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, *e.g.*, the actin promoter or an immunoglobulin promoter, from heat-shock promoters, and from the promoter normally associated with the Wnt polypeptide sequence, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication. Fiers *et al.*, *Nature* 273:113 (1978); *Mulligan et al.*; Science, 209: 1422-1427 [1980]; *Pavlakis et al.*, Proc. Natl. Acad. Sci. USA, 78: 7398-7402 [1981]. The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. *Greenaway et al.*, Gene, 18: 355-360 [1982]. A system for expressing DNA in mammalian hosts

using the bovine papilloma virus as a vector is disclosed in U.S. Patent No. 4,419,446. A modification of this system is described in U.S. Patent No. 4,601,978. See also *Gray et al.*, Nature, 295: 503-508 [1982] on expressing cDNA encoding immune interferon in monkey cells; *Reyes et al.*, Nature, 297: 598-601 [1982] on expression of human β-interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus; *Canaani et al.*, Proc. Natl. Acad. Sci. USA, 79: 5166-5170 [1982] on expression of the human interferon β1 gene in cultured mouse and rabbit cells; and *Gorman et al.*, Proc. Natl. Acad. Sci. USA, 79: 6777-6781 [1982] on expression of bacterial CAT sequences in CV-1 monkey kidney cells, chicken embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long terminal repeat as a promoter.

e. ***Enhancer element component***

Transcription of a DNA encoding the Wnt polypeptide useful in hematopoiesis according to the invention by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent, having been found 5' (*Laimins et al.*, Proc. Natl. Acad. Sci. USA, 78: 993 [1981]) and 3' (*Lusky et al.*, Mol. Cell Bio., 3: 1108 [1983]) to the transcription unit, within an intron (*Banerji et al.*, Cell, 33: 729 [1983]), as well as within the coding sequence itself. *Osborne et al.*, Mol. Cell Bio., 4: 1293 [1984]). Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α-fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, *Nature* 297:17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the Wnt polypeptide-encoding sequence, but is preferably located at a site 5' from the promoter.

f. ***Transcription termination component***

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding Wnt polypeptide.

g. ***Construction and analysis of vectors***

Construction of suitable vectors containing one or more of the above-listed components employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and re-ligated in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are used to transform *E. coli* K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction

endonuclease digestion, and/or sequenced by the method of *Messing et al.*, Nucleic Acids Res., 9: 309 [1981] or by the method of *Maxam et al.*, Methods in Enzymology, 65: 499 [1980].

h. ***Transient expression vectors***

Particularly useful in the practice of this invention are expression vectors that provide for the transient expression in mammalian cells of DNA encoding Wnt polypeptide. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector. *Sambrook et al., supra*, pp. 16.17 - 16.22. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Thus, transient expression systems are particularly useful in the invention for purposes of identifying analogs and variants of Wnt polypeptide that are biologically active Wnt polypeptide.

i. ***Suitable exemplary vertebrate cell vectors***

Other methods, vectors, and host cells suitable for adaptation to the synthesis of Wnt polypeptide in recombinant vertebrate cell culture are described in Gething *et al.*, *Nature* 293:620-625 (1981); *Mantei et al.*, Nature, 281: 40-46 [1979]; EP 117,060; and EP 117,058. A particularly useful plasmid for mammalian cell culture expression of Wnt polypeptide is pRK5 (EP 307,247) or pSVI6B. WO 91/08291 published 13 June 1991.

3. Selection and Transformation of Host Cells

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia, e.g., E. coli, Enterobacter, Erwinia, Klebsiella, Proteus, Salmonella, e.g., Salmonella typhimurium, Serratia, e.g., Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (*e.g., B. licheniformis* 41P disclosed in DD 266,710 published 12 April 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting. Strain W3110 is a particularly preferred host or parent host because it is a common host strain for recombinant DNA product fermentations. Preferably, the host cell should secrete minimal amounts of proteolytic enzymes. For example, strain W3110 may be modified to effect a genetic mutation in the genes encoding proteins, with examples of such hosts including *E. coli* W3110 strain 27C7. The complete genotype of 27C7 is *tonAΔ ptr3 phoAΔE15 Δ(argF-lac)169 ompTΔ degP41kan*^{r}. Strain 27C7 was deposited on 30 October 1991 in the American Type Culture Collection as ATCC No. 55,244. Alternatively, the strain of *E. coli* having mutant periplasmic protease disclosed in U.S. Patent No. 4,946,783 issued 7 August 1990 may be employed. Alternatively still, methods of cloning, *e.g.*, PCR or other nucleic acid polymerase reactions, are suitable. In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for Wnt polypeptide-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number

of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe* (*Beach et al.*, Nature, 290: 140 [1981]; EP 139,383 published 2 May 1985); *Kluyveromyces* hosts (U.S. Patent No. 4,943,529; *Fleer et al., supra*) such as, *e.g., K. lactis* (MW98-8C, CBS683, CBS4574), *K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickeramii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilarum* (ATCC 36,906; *Van den Berg et al., supra*), *K. thermotolerans*, and *K. marxianus; yarrowia* (EP 402,226); *Pichia pastoris* (EP 183,070; Sreekrishna *et al., J. Basic Microbiol.* 28:265-278 (1988)); *Candida; Trichoderma reesia* (EP 244,234); *Neurospora crassa* (Case *et al., Proc. Natl. Acad. Sci. USA* 76:5259-5263 (1979)); *Schwanniomyces* such as *Schwanniomyces occidentalis* (EP 394,538 published 31 October 1990); and filamentous fungi such as, *e.g., Neurospora, Penicillium, Tolypocladium* (WO 91/00357 published 10 January 1991), and *Aspergillus* hosts such as *A. nidulans* (Ballance *et al., Biochem. Biophys. Res. Commun.* 112:284-289 (1983); Tilburn *et al., Gene* 26:205-221 (1983); *Yelton et al.*, Proc. Natl. Acad. Sci. USA, 81: 1470-1474 [1984]) and *A. niger*. *Kelly et al.*, EMBO J., 4: 475-479 [1985].

Suitable host cells for the expression of glycosylated Wnt polypeptide are derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. See, *e.g., Luckow et al.*, Bio/Technology, 6: 47-55 [1988]; *Miller et al.*, in Genetic Engineering, *Setlow et al.*, eds., Vol. 8 (Plenum Publishing, 1986), pp. 277-279; and *Maeda et al.*, Nature, 315: 592-594 [1985]. A variety of viral strains for transfection are publicly available, *e.g.*, the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells.

Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can be utilized as hosts. Typically, plant cells are transfected by incubation with certain strains of the bacterium *Agrobacterium tumefaciens*, which has been previously manipulated to contain the Wnt polypeptide-encoding DNA. During incubation of the plant cell culture with *A. tumefaciens*, the DNA encoding the Wnt polypeptide is transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express the Wnt polypeptide-encoding DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences. *Depicker et al.*, J. Mol. Appl. Gen., 1: 561 [1982]. In addition, DNA segments isolated from the upstream region of the T-DNA *780* gene are capable of activating or increasing transcription levels of plant-expressible genes in recombinant DNA-containing plant tissue. EP 321,196 published 21 June 1989.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. See, *e.g., Tissue Culture*, Academic Press, Kruse and Patterson, editors (1973). Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, *Graham et al.*, J. Gen Virol., 36: 59 [1977]); baby hamster kidney cells (BHK, ATCC CCL

10); Chinese hamster ovary cells/-DHFR (CHO, *Urlaub et al.*, Proc. Natl. Acad. Sci. USA, 77: 4216 [1980]); mouse sertoli cells (TM4, *Mather*, Biol. Reprod., 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (*Mather et al.*, Annals N.Y. Acad. Sci., 383: 44-68 [1982]); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

Host cells are transfected and preferably transformed with the above-described expression or cloning vectors for Wnt polypeptide production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, $CaPO_4$ and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in section 1.82 of Sambrook *et al.*, *supra*, or electroporation is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by *Shaw et al.*, Gene, 23:315 (1983) and WO 89/05859 published 29 June 1989. In addition, plants may be transfected using ultrasound treatment as described in WO 91/00358 published 10 January 1991.

For mammalian cells without such cell walls, the calcium phosphate precipitation method of *Graham et al.*, Virology, 52: 456-457 [1978] is preferred. General aspects of mammalian cell host system transformations have been described in U.S. Pat. No. 4,399,216 issued 16 August 1983. Transformations into yeast are typically carried out according to the method of Van *Solingen et al.*, J. Bact., 130:946 [1977] and *Hsiao et al.*, Proc. Natl. Acad. Sci. USA, 76: 3829 [1979]. However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial protoplast fusion with intact cells, or polycations, *e.g.*, polybrene, polyornithine, *etc.*, may also be used. For various techniques for transforming mammalian cells, see *Keown et al.*, Methods in Enzymology, 185: 527-537 [1990] and *Mansour et al.*, Nature, 336: 348-352 [1988].

4. Culturing the Host Cells

Prokaryotic cells used to produce the Wnt polypeptide useful according to the invention are cultured in suitable media as described generally in *Sambrook et al.*, *supra*.

The mammalian host cells used to produce the Wnt polypeptide of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in *Ham et al.*, Meth. Enz., 58: 44

[1979], *Barnes et al.*, Anal. Biochem., 102: 255 [1980], U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Patent Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

In general, principles, protocols, and practical techniques for maximizing the productivity of mammalian cell cultures can be found in *Mammalian Cell Biotechnology: a Practical Approach*, M. Butler, ed. (IRL Press, 1991).

The host cells referred to in this disclosure encompass cells in culture as well as cells that are within a host animal.

5. Detecting Gene Amplification/Expression

Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA (*Thomas*, Proc. Natl. Acad. Sci. USA, 77: 5201-5205 [1980]), dot blotting (DNA analysis), or *in situ* hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, particularly ^{32}P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionuclides, fluorescers, enzymes, or the like. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, luminescent labels, and the like. A particularly sensitive staining technique suitable for use in the present invention is described by *Hsu et al.*, Am. J. Clin. Path., 75: 734-738 [1980].

Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared as described herein.

6. Purification of Wnt Polypeptide

Wnt polypeptide may be recovered from the culture medium as a secreted polypeptide, although it is preferentially recovered from host cell lysates. If the Wnt polypeptide is membrane-bound, it can be released from the cell surface using suitable agents, including enzyms or detergents (*e.g.* Triton-X 100), for example, suramin, PMA, heparin, Heparinase I and III, plasmin, n-Octyl-beta-D-glucoside, PI-specific- and PC-specific-phospholipase C and TNF-alpha.

When Wnt polypeptide is produced in a recombinant cell other than one of human origin, the Wnt polypeptide is completely free of proteins or polypeptides of human origin. However, it is necessary to purify Wnt polypeptide from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to Wnt polypeptide. As a first step, the culture medium or lysate is centrifuged to remove particulate cell debris. Wnt polypeptide thereafter is purified from contaminant soluble proteins and polypeptides, with the following procedures being exemplary of suitable purification procedures: by fractionation on an ion-exchange column; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75™; and protein A Sepharose™ columns to remove contaminants such as IgG.

Wnt polypeptide variants in which residues have been deleted, inserted, or substituted are recovered in the same fashion as native sequence Wnt polypeptide, taking account of any substantial changes in properties occasioned by the variation. Immunoaffinity columns such as a rabbit polyclonal anti-Wnt polypeptide column can be employed to absorb the Wnt polypeptide variant by binding it to at least one remaining immune epitope.

A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification, and antibiotics may be included to prevent the growth of adventitious contaminants.

7. Covalent Modifications

Covalent modifications of Wnt polypeptide are included within the scope of this invention. Both native sequence Wnt polypeptide and amino acid sequence variants of the Wnt polypeptide may be covalently modified. One type of covalent modification of the Wnt polypeptide is introduced into the molecule by reacting targeted amino acid residues of the Wnt polypeptide with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues of the Wnt polypeptide.

Cysteinyl residues most commonly are reacted with α-haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, α-bromo-β-(5-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with diethylpyrocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful; the reaction is preferably performed in 0.1M sodium cacodylate at pH 6.0.

Lysinyl and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing α-amino-containing residues include imidoesters such as methyl picolinimidate, pyridoxal phosphate, pyridoxal, chloroborohydride, trinitrobenzenesulfonic acid, O-methylisourea, 2,4-pentanedione, and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed under alkaline conditions because of the high pK_a of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as with the arginine epsilon-amino group.

The specific modification of tyrosyl residues may be made, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidizole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Tyrosyl residues are iodinated using ^{125}I or ^{131}I to prepare labeled proteins for use in radioimmunoassay, the chloramine T method being suitable.

Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides (R-N=C=N-R'), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Derivatization with bifunctional agents is useful for crosslinking Wnt polypeptide to a water-insoluble support matrix or surface for use in the method for purifying anti-Wnt polypeptide antibodies, and vice-versa. Commonly used crosslinking agents include, *e.g.*, 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidylesters such as 3,3'-dithiobis(succinimidylpropionate)and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-((p-azidophenyl)dithio)propioimidate yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Patent Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues, respectively. These residues are deamidated under neutral or basic conditions. The deamidated form of these residues falls within the scope of this invention.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the α-amino groups of lysine, arginine, and histidine side chains (T.E. Creighton, *Proteins: Structure and Molecular Properties*, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)), acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

Another type of covalent modification of the Wnt polypeptide included within the scope of this invention comprises altering the native glycosylation pattern of the polypeptide. By altering is meant deleting

one or more carbohydrate moieties found in native Wnt polypeptide, and/or adding one or more glycosylation sites that are not present in the native Wnt polypeptide.

Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serineand asparagine-X-threonine,where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxylaminoacid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the Wnt polypeptide is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-describedtripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the native Wnt polypeptide sequence (for O-linked glycosylation sites). For ease, the Wnt polypeptide amino acid sequence is preferably altered through changes at the DNA level, particularly by mutating the DNA encoding the Wnt polypeptide at preselected bases such that codons are generated that will translate into the desired amino acids. The DNA mutation(s)may be made using methods described above and in U.S. Pat. No. 5,364,934, *supra*.

Another means of increasing the number of carbohydrate moieties on the Wnt polypeptide is by chemical or enzymatic coupling of glycosides to the polypeptide. These procedures are advantageous in that they do not require production of the polypeptide in a host cell that has glycosylation capabilities for N- or O-linked glycosylation. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO 87/05330 published 11 September 1987, and in Aplin *et al., CRC Crit. Rev. Biochem.* 259-306 (1981).

Removal of carbohydrate moieties present on the Wnt polypeptide may be accomplished chemically or enzymatically. Chemical deglycosylation requires exposure of the polypeptide to the compound trifluoromethanesulfonicacid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamineor N-acetylgalactosamine),while leaving the polypeptide intact. Chemical deglycosylation is described by Hakimuddin, *et al., Arch. Biochem. Biophys.* 259:52 (1987) and by Edge *et al., Anal. Biochem.* 118:131 (1981). Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura *et al., Meth. Enzymol.* 138:350 (1987).

Glycosylation at potential glycosylation sites may be prevented by the use of the compound tunicamycin as described by Duskin *et al., J. Biol. Chem.* 257:3105 (1982). Tunicamycin blocks the formation of protein-N-glycoside linkages.

Another type of covalent modification of Wnt polypeptide comprises linking the Wnt polypeptide to one of a variety of nonproteinaceous polymers, *e.g.*, polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

Since it is often difficult to predict in advance the characteristics of a variant Wnt polypeptide, it will be appreciated that some screening of the recovered variant will be needed to select the optimal variant. A change in the immunological character of the Wnt polypeptide molecule, such as affinity for a given antibody, is also able to be measured by a competitive-type immunoassay. The Wnt polypeptide variant is assayed for changes in the ability of the protein to induce cell proliferation in the assays of Example 2. Other potential modifications of protein or polypeptide properties such as redox or thermal stability, hydrophobicity, susceptibility to proteolytic degradation, or the tendency to aggregate with carriers or into multimers are assayed by methods well known in the art.

8. Epitope-Tagged Wnt Polypeptide

This invention encompasses chimeric polypeptides comprising Wnt polypeptide fused to a heterologous polypeptide. A chimeric Wnt polypeptide is one type of Wnt polypeptide variant as defined herein. In one preferred embodiment, the chimeric polypeptide comprises a fusion of the Wnt polypeptide with a tag polypeptide which provides an epitope to which an anti-tag antibody can selectively bind. The epitope tag is generally provided at the amino- or carboxyl- terminus of the Wnt polypeptide. Such epitope-tagged forms of the Wnt polypeptide are desirable as the presence thereof can be detected using a labeled antibody against the tag polypeptide. Also, provision of the epitope tag enables the Wnt polypeptide to be readily purified by affinity purification using the anti-tag antibody. Affinity purification techniques and diagnostic assays involving antibodies are described later herein. Tag polypeptides and their respective antibodies are well known in the art. Examples include the Herpes Simplex virus glycoprotein D (gD) tag and its antibody (*Pennica et al., supra*); the his tag, for example his_6 (*Hengen*, Trends Biochem. Sci., 20: 285-286 [1995] and *Pennica et al.*, J. Biol. Chem., 270: 10915-10922 [1995]); the flu HA tag polypeptide and its antibody 12CA5 (*Field et al.*, Mol. Cell. Biol., 8: 2159-2165 [1988]); the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto (*Evan et al.*, Molecular and Cellular Biology, 5: 3610-3616 [1985]; and *Paborsky et al.*, Protein Engineering, 3(6): 547-553 [1990]). Other tag polypeptides have been disclosed. Examples include the Flag-peptide (*Hopp et al.*, BioTechnology, 6: 1204-1210 [1988]); the KT3 epitope peptide (*Martin et al.*, Science, 255: 192-194 [1992]); an α-tubulin epitope peptide (*Skinner et al.*, J. Biol. Chem., 266: 15163-15166 [1991]); and the T7 gene 10 protein peptide tag. *Lutz-Freyermuth et al.*, Proc. Natl. Acad. Sci. USA, 87: 6393-6397 [1990]. Once the tag polypeptide has been selected, an antibody thereto can be generated using the techniques disclosed herein.

The general methods suitable for the construction and production of epitope-tagged Wnt polypeptide are the same as those disclosed hereinabove. Wnt polypeptide-tag polypeptide fusions are most conveniently constructed by fusing the cDNA sequence encoding the Wnt polypeptide portion in-frame to the tag polypeptide DNA sequence and expressing the resultant DNA fusion construct in appropriate host cells. Ordinarily, when preparing the Wnt polypeptide-tag polypeptide chimeras of the present invention, nucleic acid encoding the Wnt

polypeptide will be fused at its 3' end to nucleic acid encoding the N-terminus of the tag polypeptide, however 5' fusions are also possible.

Epitope-tagged Wnt polypeptide can be conveniently purified by affinity chromatography using the anti-tag antibody. The matrix to which the affinity antibody is attached is most often agarose, but other matrices are available (*e.g.* controlled pore glass or poly(styrenedivinyl)benzene). The epitope-tagged Wnt polypeptide can be eluted from the affinity column by varying the buffer pH or ionic strength or adding chaotropic agents, for example.

9. Wnt Polypeptide Immunoadhesins

Chimeras constructed from a receptor sequence linked to an appropriate immunoglobulin constant domain sequence (immunoadhesins) are known in the art. Immunoadhesins reported in the literature include fusions of the T cell receptor* (Gascoigne *et al.*, *Proc. Natl.Acad. Sci. USA* 84: 2936-2940 (1987)); CD4* (Capon *et al.*, *Nature* 337: 525-531 (1989); Traunecker *et al.*, *Nature* 339: 68-70 (1989); Zettmeissl *et al.*, *DNA Cell Biol. USA* 9: 347-353 (1990); Byrn *et al.*, *Nature* 344: 667-670 (1990)); L-selectin (homing receptor) ((Watson *et al.*, *J. Cell. Biol.* 110:2221-2229 (1990); Watson *et al.*, *Nature* 349: 164-167 (1991)); CD44* (Aruffo *et al.*, *Cell* 61: 1303-1313 (1990)); CD28* and B7* (Linsley *et al.*, *J. Exp. Med.* 173: 721-730 (1991)); CTLA-4* (Lisley *et al.*, *J. Exp. Med.* 174: 561-569 (1991)); CD22* (Stamenkovic *et al.*, *Cell* 66:1133-1144 (1991)); TNF receptor (Ashkenazi *et al.*, *Proc. Natl. Acad. Sci. USA* 88: 10535-10539 (1991); Lesslauer *et al.*, *Eur. J. Immunol.* 27: 2883-2886 (1991); Peppel *et al.*, *J. Exp. Med.* 174:1483-1489 (1991)); NP receptors (Bennett *et al.*, *J. Biol. Chem.* 266:23060-23067 (1991)); and IgE receptor α* (Ridgway *et al.*, *J. Cell. Biol.* 115:abstr. 1448 (1991)), where the asterisk (*) indicates that the receptor is member of the immunoglobulin superfamily.

The simplest and most straightforward immunoadhesin design combines the binding region(s) of the "adhesin" protein with the hinge and Fc regions of an immunoglobulin heavy chain. Ordinarily, when preparing the Wnt polypeptide-immunoglobulin chimeras of the present invention, nucleic acid encoding Wnt polypeptide will be fused C-terminally to nucleic acid encoding the N-terminus of an immunoglobulin constant domain sequence, however N-terminal fusions are also possible.

Typically, in such fusions the encoded chimeric polypeptide will retain at least functionally active hinge, CH2 and CH3 domains of the constant region of an immunoglobulin heavy chain. Fusions are also made to the C-terminus of the Fc portion of a constant domain, or immediately N-terminal to the CH1 of the heavy chain or the corresponding region of the light chain.

The precise site at which the fusion is made is not critical; particular sites are well known and may be selected in order to optimize the biological activity, secretion or binding characteristics of the Wnt polypeptide-immunoglobulin chimeras.

In some embodiments, the Wnt polypeptide-immunoglobulin chimeras are assembled as monomers, or hetero- or homo-multimers, and particularly as dimers or tetramers, essentially as illustrated in WO 91/08298.

In a preferred embodiment, the Wnt polypeptide sequence is fused to the N-terminus of the C-terminal portion of an antibody (in particular the Fc domain), containing the effector functions of an immunoglobulin, *e.g.* immunoglobulin G1 (IgG1). It is possible to fuse the entire heavy chain constant region to the Wnt

polypeptide sequence. However, more preferably, a sequence beginning in the hinge region just upstream of the papain cleavage site (which defines IgG Fc chemically; residue 216, taking the first residue of heavy chain constant region to be 114, or analogous sites of other immunoglobulins) is used in the fusion. In a particularly preferred embodiment, the Wnt polypeptide amino acid sequence is fused to the hinge region, CH2 and CH3, or the CH1, hinge, CH2 and CH3 domains of an IgG1, IgG2, or IgG3 heavy chain. The precise site at which the fusion is made is not critical, and the optimal site can be determined by routine experimentation.

In some embodiments, the Wnt polypeptide-immunoglobulin chimeras are assembled as multimers, and particularly as homo-dimers or -tetramers. Generally, these assembled immunoglobulins will have known unit structures. A basic four chain structural unit is the form in which IgG, IgD, and IgE exist. A four unit is repeated in the higher molecular weight immunoglobulins; IgM generally exists as a pentamer of basic four units held together by disulfide bonds. IgA globulin, and occasionally IgG globulin, may also exist in multimeric form in serum. In the case of multimer, each four unit may be the same or different.

Various exemplary assembled Wnt polypeptide-immunoglobulinchimeras within the scope herein are schematically diagrammed below:

(a) AC_L-AC_L;

(b) AC_H-(AC_H, AC_L-AC_H, AC_L-V_HC_H, or V_LC_L-AC_H);

(c) AC_L-AC_H-(AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, or V_LC_L-V_HC_H);

(d) AC_L-V_HC_H-(AC_H, or AC_L-V_HC_H, or V_LC_L-AC_H);

(e) V_LC_L-AC_H-(AC_L-V_HC_H, or V_LC_L-AC_H); and

(f) $(A\text{-}Y)_n$-$(V_LC_L\text{-}V_HC_H)_2$,

wherein

each A represents identical or different Wnt polypeptide or amino acid sequences;

V_L is an immunoglobulin light chain variable domain;

V_H is an immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain;

C_H is an immunoglobulin heavy chain constant domain;

n is an integer greater than 1;

Y designates the residue of a covalent cross-linking agent.

In the interests of brevity, the foregoing structures only show key features; they do not indicate joining (J) or other domains of the immunoglobulins, nor are disulfide bonds shown. However, where such domains are required for binding activity, they shall be constructed as being present in the ordinary locations which they occupy in the immunoglobulin molecules.

Alternatively, the Wnt polypeptide sequence can be inserted between immunoglobulin heavy chain and light chain sequences such that an immunoglobulin comprising a chimeric heavy chain is obtained. In this embodiment, the Wnt polypeptide sequence is fused to the 3' end of an immunoglobulin heavy chain in each arm of an immunoglobulin, either between the hinge and the CH2 domain, or between the CH2 and CH3 domains. Similar constructs have been reported by Hoogenboom *et al.*, *Mol. Immunol.*, 28:1027-1037 (1991).

Although the presence of an immunoglobulin light chain is not required in the immunoadhesins of the present invention, an immunoglobulin light chain might be present either covalently associated to an Wnt polypeptide-immunoglobulinheavy chain fusion polypeptide, or directly fused to the Wnt polypeptide. In the former case, DNA encoding an immunoglobulin light chain is typically coexpressed with the DNA encoding the Wnt polypeptide-immunoglobulinheavy chain fusion protein. Upon secretion, the hybrid heavy chain and the light chain will be covalently associated to provide an immunoglobulin-like structure comprising two disulfide-linked immunoglobulin heavy chain-light chain pairs. Methods suitable for the preparation of such structures are, for example, disclosed in U.S. Patent No. 4,816,567 issued 28 March 1989.

In a preferred embodiment, the immunoglobulin sequences used in the construction of the immunoadhesins of the present invention are from an IgG immunoglobulin heavy chain constant domain. For human immunoadhesins, the use of human IgG1 and IgG3 immunoglobulin sequences is preferred. A major advantage of using IgG1 is that IgG1 immunoadhesins can be purified efficiently on immobilized protein A. In contrast, purification of IgG3 requires protein G, a significantly less versatile medium. However, other structural and functional properties of immunoglobulins should be considered when choosing the Ig fusion partner for a particular immunoadhesin construction. For example, the IgG3 hinge is longer and more flexible, so it can accommodate larger adhesin domains that may not fold or function properly when fused to IgG1. Another consideration may be valency; IgG immunoadhesins are bivalent homodimers, whereas Ig subtypes like IgA and IgM may give rise to dimeric or pentameric structures, respectively, of the basic Ig homodimer unit. For immunoadhesins designed for *in vivo* application, the pharmacokinetic properties and the effector functions specified by the Fc region are important as well. Although IgG1, IgG2 and IgG4 all have *in vivo* half-lives of 21 days, their relative potencies at activating the complement system are different. IgG4 does not activate complement, and IgG2 is significantly weaker at complement activation than IgG1. Moreover, unlike IgG1, IgG2 does not bind to Fc receptors on mononuclear cells or neutrophils. While IgG3 is optimal for complement activation, its *in vivo* half-life is approximately one third of the other IgG isotypes. Another important consideration for immunoadhesins designed to be used as human therapeutics is the number of allotypic variants of the particular isotype. In general, IgG isotypes with fewer serologically-defined allotypes are preferred. For example, IgG1 has only four serologically-defined allotypic sites, two of which (G1m and 2) are located in the Fc region; and one of these sites G1m1, is non-immunogenic. In contrast, there are 12 serologically-defined allotypes in IgG3, all of which are in the Fc region; only three of these sites (G3m5, 11 and 21) have one allotype which is nonimmunogenic. Thus, the potential immunogenicity of a γ3 immunoadhesin is greater than that of a γ1 immunoadhesin.

With respect to the parental immunoglobulin, a useful joining point is just upstream of the cysteines of the hinge that form the disulfide bonds between the two heavy chains. In a frequently used design, the codon for the C-terminal residue of the Wnt polypeptide part of the molecule is placed directly upstream of the codons for the sequence DKTHTCPPCP (SEQ ID NO: 1) of the IgG1 hinge region.

The general methods suitable for the construction and expression of immunoadhesins are the same as those disclosed hereinabove with regard to Wnt polypeptide. Immunoadhesins are most conveniently constructed by fusing the cDNA sequence encoding the Wnt polypeptide portion in-frame to an Ig cDNA

sequence. However, fusion to genomic Ig fragments can also be used (see, *e.g.*, Gascoigne *et al.*, *Proc. Natl. Acad. Sci. USA*, 84:2936-2940 (1987); Aruffo *et al.*, *Cell* 61:1303-1313 (1990); Stamenkovic *et al.*, *Cell* 66:1133-1144 (1991)). The latter type of fusion requires the presence of Ig regulatory sequences for expression. cDNAs encoding IgG heavy-chain constant regions can be isolated based on published sequence from cDNA libraries derived from spleen or peripheral blood lymphocytes, by hybridization or by polymerase chain reaction (PCR) techniques. The cDNAs encoding the Wnt polypeptide and Ig parts of the immunoadhesin are inserted in tandem into a plasmid vector that directs efficient expression in the chosen host cells. For expression in mammalian cells, pRK5-based vectors (Schall *et al.*, *Cell* 61:361-370 (1990)) and CDM8-based vectors (Seed, *Nature* 329:840 (1989)) can be used. The exact junction can be created by removing the extra sequences between the designed junction codons using oligonucleotide-directed deletional mutagenesis (Zoller *et al.*, *Nucleic Acids Res.* 10:6487 (1982); Capon *et al.*, *Nature* 337:525-531 (1989)). Synthetic oligonucleotides can be used, in which each half is complementary to the sequence on either side of the desired junction; ideally, these are 36 to 48-mers. Alternatively, PCR techniques can be used to join the two parts of the molecule in-frame with an appropriate vector.

The choice of host cell line for the expression of the immunoadhesin depends mainly on the expression vector. Another consideration is the amount of protein that is required. Milligram quantities often can be produced by transient transfections. For example, the adenovirus EIA-transformed 293 human embryonic kidney cell line can be transfected transiently with pRK5-based vectors by a modification of the calcium phosphate method to allow efficient immunoadhesin expression. CDM8-based vectors can be used to transfect COS cells by the DEAE-dextran method (Aruffo *et al.*, *Cell* 61:1303-1313 (1990); Zettmeissl *et al.*, *DNA Cell Biol. US* 9:347-353 (1990)). If larger amounts of protein are desired, the immunoadhesin can be expressed after stable transfection of a host cell line. For example, a pRK5-based vector can be introduced into Chinese hamster ovary (CHO) cells in the presence of an additional plasmid encoding dihydrofolate reductase (DHFR) and conferring resistance to G418. Clones resistant to G418 can be selected in culture; these clones are grown in the presence of increasing levels of DHFR inhibitor methotrexate; clones are selected, in which the number of gene copies encoding the DHFR and immunoadhesin sequences is co-amplified. If the immunoadhesin contains a hydrophobic leader sequence at its N-terminus, it is likely to be processed and secreted by the transfected cells. The expression of immunoadhesins with more complex structures may require uniquely suited host cells; for example, components such as light chain or J chain may be provided by certain myeloma or hybridoma cell hosts (Gascoigne *et al.*, 1987, *supra*, Martin *et al.*, *J. Virol.* 67:3561-3568 (1993)).

Immunoadhesins can be conveniently purified by affinity chromatography. The suitability of protein A as an affinity ligand depends on the species and isotype of the immunoglobulin Fc domain that is used in the chimera. Protein A can be used to purify immunoadhesins that are based on human γ1, γ2, or γ4 heavy chains (Lindmark *et al.*, *J. Immunol. Meth.* 62:1-13 (1983)). Protein G is recommended for all mouse isotypes and for human γ3 (Guss *et al.*, *EMBO J.* 5:1567-1575 (1986)). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or *poly*(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. The conditions for binding an immunoadhesin to the protein A or G affinity column are dictated

entirely by the characteristics of the Fc domain; that is, its species and isotype. Generally, when the proper ligand is chosen, efficient binding occurs directly from unconditioned culture fluid. One distinguishing feature of immunoadhesins is that, for human γ1 molecules, the binding capacity for protein A is somewhat diminished relative to an antibody of the same Fc type. Bound immunoadhesin can be efficiently eluted either at acidic pH (at or above 3.0), or in a neutral pH buffer containing a mildly chaotropic salt. This affinity chromatography step can result in an immunoadhesin preparation that is >95% pure.

Other methods known in the art can be used in place of, or in addition to, affinity chromatography on protein A or G to purify immunoadhesins. Immunoadhesins behave similarly to antibodies in thiophilic gel chromatography (Hutchens *et al.*, *Anal. Biochem.* 159:217-226 (1986)) and immobilized metal chelate chromatography (Al-Mashikhi *et al.*, *J. Dairy Sci.* 71:1756-1763 (1988)). In contrast to antibodies, however, their behavior on ion exchange columns is dictated not only by their isoelectric points, but also by a charge dipole that may exist in the molecules due to their chimeric nature.

If desired, the immunoadhesins can be made bispecific. Thus, the immunoadhesins of the present invention may combine a Wnt polypeptide and a domain, such as the extracellular domain, of another cytokine receptor subunit. Exemplary cytokine receptors from which such bispecific immunoadhesin molecules can be made include TPO (or *mpl* ligand), EPO, G-CSF, IL-4, IL-7, GH, PRL, IL-3, GM-CSF, IL-5, IL-6, LIF, OSM, CNTF and IL-2 receptors. Alternatively, a Wnt polypeptide may be combined with another cytokine, such as those exemplified herein, in the generation of a bispecific immunoadhesin. For bispecific molecules, trimeric molecules, composed of a chimeric antibody heavy chain in one arm and a chimeric antibody heavy chain-light chain pair in the other arm of their antibody-like structure are advantageous, due to ease of purification. In contrast to antibody-producing quadromas traditionally used for the production of bispecific immunoadhesins, which produce a mixture of ten tetramers, cells transfected with nucleic acid encoding the three chains of a trimeric immunoadhesin structure produce a mixture of only three molecules, and purification of the desired product from this mixture is correspondingly easier.

10. Long Half-Life Derivatives of Wnt Polypeptides

Wnt polypeptide functional derivatives for use in the methods of the present invention include Wnt-immunoglobulin chimeras (immunoadhesins) and other longer half-life molecules. Other derivatives of the Wnt polypeptides, which possess a longer half-life than the native molecules comprise the Wnt polypeptide or a Wnt-immunoglobulin chimera covalently bonded to a nonproteinaceous polymer. The nonproteinaceous polymer ordinarily is a hydrophilic synthetic polymer, *i.e.*, a polymer not otherwise found in nature. However, polymers which exist in nature and are produced by recombinant or *in vitro* methods are useful, as are polymers which are isolated from native sources. Hydrophilic polyvinyl polymers fall within the scope of this invention, *e.g.* polyvinylalcohol and polyvinylpyrrolidone. Particularly useful are polyalkylene ethers such as polyethylene glycol (PEG); polyelkylenes such as polyoxyethylene, polyoxypropylene, and block copolymers of polyoxyethylene and polyoxypropylene (Pluronics™); polymethacrylates; carbomers; branched or unbranched polysaccharides which comprise the saccharide monomers D-mannose, D- and L-galactose, fucose, fructose, D-xylose, L-arabinose, D-glucuronic acid, sialic acid, D-galacturonic acid, D-mannuronic acid (*e.g.* polymannuronic acid, or alginic acid), D-glucosamine, D-galactosamine, D-glucose and neuraminic acid

including homopolysaccharides and heteropolysaccharides such as lactose, amylopectin, starch, hydroxyethyl starch, amylose, dextrane sulfate, dextran, dextrins, glycogen, or the polysaccharide subunit of acid mucopolysaccharides, *e.g.* hyaluronic acid; polymers of sugar alcohols such as polysorbitol and polymannitol; heparin or heparon. The polymer prior to cross-linking need not be, but preferably is, water soluble, but the final conjugate must be water soluble. In addition, the polymer should not be highly immunogenic in the conjugate form, nor should it possess viscosity that is incompatible with intravenous infusion or injection if it is intended to be administered by such routes.

Preferably the polymer contains only a single group which is reactive. This helps to avoid cross-linking of protein molecules. However, it is within the scope herein to optimize reaction conditions to reduce cross-linking, or to purify the reaction products through gel filtration or chromatographic sieves to recover substantially homogenous derivatives.

The molecular weight of the polymer may desirably range from about 100 to 500,000, and preferably is from about 1,000 to 20,000. The molecular weight chosen will depend upon the nature of the polymer and the degree of substitution. In general, the greater the hydrophilicity of the polymer and the greater the degree of substitution, the lower the molecular weight that can be employed. Optimal molecular weights will be determined by routine experimentation.

The polymer generally is covalently linked to the Wnt polypeptide or to the Wnt-immunoglobulin chimera though a multifunctional crosslinking agent which reacts with the polymer and one or more amino acid or sugar residues of the Wnt polypeptide or Wnt-immunoglobulin chimera to be linked. However, it is within the scope of the invention to directly crosslink the polymer by reacting a derivatized polymer with the hybrid, or via versa.

The covalent crosslinking site on the Wnt polypeptide or Wnt-immunoglobulin chimera includes the N-terminal amino group and epsilon amino groups found on lysine residues, as well as other amino, imino, carboxyl, sulfhydryl, hydroxyl or other hydrophilic groups. The polymer may be covalently bonded directly to the hybrid without the use of a multifunctional (ordinarily bifunctional) crosslinking agent. Covalent binding to amino groups is accomplished by known chemistries based upon cyanuric chloride, carbonyl diimidazole, aldehyde reactive groups (PEG alkoxide plus diethyl acetal of bromoacetaldehyde; PEG plus DMSO and acetic anhydride, or PEG chloride plus the phenoxide of 4-hydroxybenzaldehyde, succinimidyl active esters, activated dithiocarbonate PEG, 2,4,5-trichlorophenylcloroformate or P-nitrophenylcloroformate activated PEG). Carboxyl groups are derivatized by coupling PEG-amine using carbodiimide.

Polymers are conjugated to oligosaccharide groups by oxidation using chemicals, *e.g.* metaperiodate, or enzymes, *e.g.* glucose or galactose oxidase (either of which produces the aldehyde derivative of the carbohydrate), followed by reaction with hydrazide or amino derivatized polymers, in the same fashion as is described by Heitzmann *et al.*, *P.N.A.S.* 71:3537-41 (1974) or Bayer *et al.*, *Methods in Enzymology* 62:310 (1979), for the labeling of oligosaccharides with biotin or avidin. Further, other chemical or enzymatic methods which have been used heretofore to link oligosaccharides are particularly advantageous because, in general, there are fewer substitutions than amino acid sites for derivatization, and the oligosaccharide products thus will

be more homogenous. The oligosaccharide substituents also are optionally modified by enzyme digestion to remove sugars, *e.g.* by neuraminidase digestion, prior to polymer derivatization.

The polymer will bear a group which is directly reactive with an amino acid side chain, or the N- or C-terminus of the polypeptide linked, or which is reactive with the multifunctional cross-linking agent. In general, polymers bearing such reactive groups are known for the preparation of immobilized proteins. In order to use such chemistries here, one should employ a water soluble polymer otherwise derivatized in the same fashion as insoluble polymers heretofore employed for protein immobilization. Cyanogen bromide activation is a particularly useful procedure to employ in crosslinking polysaccharides.

"Water soluble" in reference to the starting polymer means that the polymer or its reactive intermediate used for conjugation is sufficiently water soluble to participate in a derivatization reaction.

"Water soluble" in reference to the polymer conjugate means that the conjugate is soluble in physiological fluids such as blood.

The degree of substitution with such a polymer will vary depending upon the number of reactive sites on the protein, whether all or a fragment of the protein is used, whether the protein is a fusion with a heterologous protein (*e.g.* an Wnt-immunoglobulin chimera), the molecular weight, hydrophilicity and other characteristics of the polymer, and the particular protein derivatization sites chosen. In general, the conjugate contains about from 1 to 10 polymer molecules, while any heterologous sequence may be substituted with an essentially unlimited number of polymer molecules so long as the desired activity is not significantly adversely affected. The optimal degree of cross-linking is easily determined by an experimental matrix in which the time, temperature and other reaction conditions are varied to change the degree of substitution, after which the ability of the conjugates to function in the desired fashion is determined.

The polymer, *e.g.* PEG, is cross-linked by a wide variety of methods known *per se* for the covalent modification of proteins with nonproteinaceous polymers such as PEG. Certain of these methods, however, are not preferred for the purposes herein. Cyanuronic chloride chemistry leads to many side reactions, including protein cross-linking. In addition, it may be particularly likely to lead to inactivation of proteins containing sulfhydryl groups. Carbonyl diimidazole chemistry (Beauchamp *et al.*, *Anal Biochem.* 131:25-33 (1983)) requires high pH (>8.5), which can inactivate proteins. Moreover, since the "activated PEG" intermediate can react with water, a very large molar excess of "activated PEG" over protein is required. The high concentrations of PEG required for the carbonyl diimidazole chemistry also led to problems in purification, as both gel filtration chromatography and hydrophilic interaction chromatography are adversely affected. In addition, the high concentrations of "activated PEG" may precipitate protein, a problem that *per se* has been noted previously (Davis, U.S. Patent No. 4,179,337). On the other hand, aldehyde chemistry (Royer, U.S. Patent No. 4,002,531) is more efficient since it requires only a 40-fold molar excess of PEG and a 1-2 hr incubation. However, the manganese dioxide suggested by Royer for preparation of the PEG aldehyde is problematic "because of the pronounced tendency of PEG to form complexes with metal-based oxidizing agents" (Harris *et al.*, *J. Polym. Sci. Polym. Chem. Ed.* 22:341-52 (1984)). The use of a Moffatt oxidation, utilizing DMSO and acetic anhydride, obviates this problem. In addition, the sodium borohydride suggested by Royer must be used at high

pH and has a significant tendency to reduce disulfide bonds. In contrast, sodium cyanoborohydride, which is effective at neutral pH and has very little tendency to reduce disulfide bonds is preferred.

Functionalized PEG polymers to modify the Wnt polypeptide or Wnt-immunoglobulin chimeras of the present invention are available from Shearwater Polymers, Inc. (Huntsville, AL). Such commercially available PEG derivatives include, but are not limited to, amino-PEG, PEG amino acid esters, PEG-hydrazide, PEG-thiol, PEG-succinate, carboxymethylated PEG, PEG-propionic acid, PEG amino acids, PEG succinimidyl succinate, PEG succinimidyl propionate, succinimidyl ester of carboxymethylated PEG, succinimidyl carbonate of PEG, succinimidyl esters of amino acid PEGs, PEG-oxycarbonylimidazole, PEG-nitrophenyl carbonate, PEG tresylate, PEG-glycidyl ether, PEG-aldehyde, PEG vinylsulfone, PEG-maleimide, PEG-orthopyridyl-disulfide, heterofunctional PEGs, PEG vinyl derivatives, PEG silanes, and PEG phospholides. The reaction conditions for coupling these PEG derivatives will vary depending on the protein, the desired degree of PEGylation, and the PEG derivative utilized. Some factors involved in the choice of PEG derivatives include: the desired point of attachment (lysine or cysteine), hydrolytic stability and reactivity of the derivatives, stability, toxicity and antigenicity of the linkage, suitability for analysis, etc. Specific instructions for the use of any particular derivative are available from the manufacturer.

The long half-life conjugates of this invention are separated from the unreacted starting materials by gel filtration. Heterologous species of the conjugates are purified from one another in the same fashion. The polymer also may be water-insoluble, as a hydrophilic gel.

The conjugates may also be purified by ion-exchange chromatography. The chemistry of many of the electrophilically activated PEG's results in a reduction of amino group charge of the PEGylated product. Thus, high resolution ion exchange chromatography can be used to separate the free and conjugated proteins, and to resolve species with different levels of PEGylation. In fact, the resolution of different species (*e.g.* containing one or two PEG residues) is also possible due to the difference in the ionic properties of the unreacted amino acids.

B. Therapeutic Uses for the Wnt Polypeptide

The Wnt polypeptide and Wnt polypeptide gene are believed to find therapeutic use for administration to a mammal in the treatment of diseases or disorders characterized by a decrease in hematopoietic cells. Examples of these diseases or disorders include: anemia (including macrocytic and aplastic anemia); thrombocytopenia; hypoplasia; disseminated intravascular coagulation (DIC); myelodysplasia; immune (autoimmune) thrombocytopenic purpura (ITP); and HIV induced ITP. Additionally, these Wnt polypeptide molecules may be useful in treating patients having suffered a hemorrhage. Wnt polypeptide and Wnt polypeptide gene which lead to an increase in hematopoietic cell proliferation may also be used to enhance repopulation of mature blood cell lineages in cells having undergone chemo- or radiation therapy or bone marrow transplantation therapy. Generally, these molecules are expected to lead to an enhancement of the proliferation, differentiation and/or maintenance of primitive hematopoietic cells.

The Wnt polypeptide may be administered alone or in combination with one or more cytokines, including growth factors or antibodies in the above-identified clinical situations. This may facilitate an effective

lowering of the dose of Wnt polypeptide. Suitable dosages for such additional molecules will be discussed below.

In gene therapy applications, genes are introduced into cells in order to achieve *in vivo* synthesis of a therapeutically effective genetic product, for example for replacement of a defective gene. "Gene therapy" includes both conventional gene therapy where a lasting effect is achieved by a single treatment, and the administration of gene therapeutic agents, which involves the one time or repeated administration of a therapeutically effective DNA or mRNA. Antisense RNAs and DNAs can be used as therapeutic agents for blocking the expression of certain genes *in vivo*. It has already been shown that short antisense oligonucleotides can be imported into cells where they act as inhibitors, despite their low intracellular concentrations caused by their restricted uptake by the cell membrane. (Zamecnik *et al.*, *Proc. Natl. Acad. Sci. USA*, 83:4143-4146 (1986)). The oligonucleotides can be modified to enhance their uptake, *e.g.*, by substituting their negatively charged phosphodiester groups by uncharged groups.

There are a variety of techniques available for introducing nucleic acids into viable cells. The techniques vary depending upon whether the nucleic acid is transferred into cultured cells *in vitro*, or *in vivo* in the cells of the intended host. Techniques suitable for the transfer of nucleic acid into mammalian cells *in vitro* include the use of liposomes, electroporation, microinjection, cell fusion, DEAE-dextran, the calcium phosphate precipitation method, etc. The currently preferred *in vivo* gene transfer techniques include transfection with viral (typically retroviral) vectors and viral coat protein-liposomemediated transfection (Dzau *et al.*, *Trends in Biotechnology* 11:205-210(1993)). In some situations it is desirable to provide the nucleic acid source with an agent that targets the target cells, such as an antibody specific for a cell surface membrane protein or the target cell, a ligand for a receptor on the target cell, etc. Where liposomes are employed, proteins which bind to a cell surface membrane protein associated with endocytosis may be used for targeting and/or to facilitate uptake, *e.g.* capsid proteins or fragments thereof tropic for a particular cell type, antibodies for proteins which undergo internalization in cycling, and proteins that target intracellular localization and enhance intracellular half-life. The technique of receptor-mediatedendocytosis is described, for example, by Wu *et al.*, *J. Biol. Chem.* 262:4429-4432(1987); and Wagner *et al.*, *Proc. Natl. Acad. Sci. USA* 87:3410-3414(1990). For review of the currently known gene marking and gene therapy protocols see Anderson *et al.*, *Science* 256:808-813 (1992).

For therapeutic applications, the Wnt polypeptide useful according to the invention are administered to a mammal, preferably a human, in a physiologically acceptable dosage form, including those that may be administered to a human intravenously as a bolus or by continuous infusion over a period of time. Alternative routes of administration include intramuscular, intraperitoneal, intra-cerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. The Wnt polypeptides also are suitably administered by intratumoral, peritumoral, intralesional, or perilesional routes or to the lymph, to exert local as well as systemic therapeutic effects.

Such dosage forms encompass physiologically acceptable carriers that are inherently non-toxic and non-therapeutic. Examples of such carriers include ion exchangers, alumina, aluminum stearate, lecithin, serum proteins, such as human serum albumin, buffer substances such as phosphates, glycine, sorbic acid, potassium

sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts, or electrolytes such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based substances, and PEG. Carriers for topical or gel-based forms of Wnt polypeptides include polysaccharides such as sodium carboxymethylcellulose or methylcellulose, polyvinylpyrrolidone, polyacrylates, polyoxyethylene-polyoxypropylene-blockpolymers, PEG, and wood wax alcohols. For all administrations, conventional depot forms are suitably used. Such forms include, for example, microcapsules, nano-capsules, liposomes, plasters, inhalation forms, nose sprays, sublingual tablets, and sustained-releasepreparations. The Wnt polypeptide will typically be formulated in such vehicles at a concentration of about 0.1 mg/ml to 100 mg/ml.

Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the Wnt polypeptide, which matrices are in the form of shaped articles, *e.g.* films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate) as described by Langer *et al.*, *supra* and Langer, *supra*, or poly(vinylalcohol), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate (Sidman *et al.*, *supra*), non-degradableethylene-vinylacetate (Langer *et al.*, *supra*), degradable lactic acid-glycolic acid copolymers such as the Lupron Depot™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated Wnt polypeptides remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

Sustained-release Wnt polypeptide compositions also include liposomally entrapped polypeptides. Liposomes containing the Wnt polypeptide are prepared by methods known in the art, such as described in Epstein *et al.*, *Proc. Natl. Acad. Sci. USA* 82:3688 (1985); Hwang *et al.*, *Proc. Natl. Acad. Sci. USA* 77:4030 (1980); and U.S. Pat. Nos. 4,485,045 and 4,544,545. Ordinarily, the liposomes are the small (about 200-800 Angstroms) unilamelar type in which the lipid content is greater than about 30 mol.% cholesterol, the selected proportion being adjusted for the optimal Wnt polypeptide therapy. Liposomes with enhanced circulation time are disclosed in U.S. Patent No. 5,013,556.

For the prevention or treatment of disease, the appropriate dosage of Wnt polypeptide will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antibodies are administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the Wnt polypeptide, and the discretion of the attending physician. The Wnt polypeptide is suitably *administered* to the patient at one time or over a series of treatments.

Depending on the type and severity of the disease, about 1 μg/kg to 15 mg/kg of Wnt polypeptide is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 μg/kg to 100 μg/kg (*e.g.* 1-50 μg/kg) or more, depending on the factors mentioned above. For example, the dose may be the same as that for other cytokines such as G-CSF, GM-CSF and EPO. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

When one or more cytokines are co-administered with the Wnt polypeptide, lesser doses of the Wnt polypeptide may be employed. Suitable doses of a cytokine are from about 1μg/kg to about 15mg/kg of cytokine. A typical daily dosage of the cytokine might range from about 1 μg/kg to 100 μg/kg (*e.g.* 1-50 μg/kg) or more. For example, the dose may be the same as that for other cytokines such as G-CSF, GM-CSF and EPO. The cytokine(s) may be administered prior to, simultaneously with, or following administration of the Wnt polypeptide. The cytokine(s) and Wnt polypeptide may be combined to form a pharmaceutically composition for simultaneous administration to the mammal. In certain embodiments, the amounts of Wnt polypeptide and cytokine are such that a synergistic repopulation of blood cells (or synergistic increase in proliferation and/or differentiation of hematopoietic cells) occurs in the mammal upon administration of the Wnt polypeptide and cytokine thereto. In other words, the coordinated action of the two or more agents (*i.e.* the Wnt polypeptide and cytokine(s)) with respect to repopulation of blood cells (or proliferation/differentiation of hematopoietic cells) is greater than the sum of the individual effects of these molecules.

Therapeutic formulations of Wnt polypeptide are prepared for storage by mixing Wnt polypeptide having the desired degree of purity with optional physiologically acceptable carriers, excipients, or stabilizers (*Remington's Pharmaceutical Sciences*, 16th edition, Osol, A., Ed., (1980)), in the form of lyophilized cake or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counter-ions such as sodium; and/or non-ionic surfactants such as Tween, Pluronics™ or polyethylene glycol (PEG).

The Wnt polypeptide also may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (for example, hydroxymethylcelluloseor gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively), in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles, and nanocapsules), or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences, supra.*

Wnt polypeptide to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to or following lyophilization and reconstitution. Wnt

polypeptide ordinarily will be stored in lyophilized form or in solution. Therapeutic Wnt polypeptide compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

When applied topically, the Wnt polypeptide is suitably combined with other ingredients, such as carriers and/or adjuvants. There are no limitations on the nature of such other ingredients, except that they must be physiologically acceptable and efficacious for their intended administration, and cannot degrade the activity of the active ingredients of the composition. Examples of suitable vehicles include ointments, creams, gels, or suspensions, with or without purified collagen. The compositions also may be impregnated into transdermal patches, plasters, and bandages, preferably in liquid or semi-liquid form.

For obtaining a gel formulation, the Wnt polypeptide formulated in a liquid composition may be mixed with an effective amount of a water-soluble polysaccharide or synthetic polymer such as PEG to form a gel of the proper viscosity to be applied topically. The polysaccharide that may be used includes, for example, cellulose derivatives such as etherified cellulose derivatives, including alkyl celluloses, hydroxyalkyl celluloses, and alkylhydroxyalkyl celluloses, for example, methylcellulose, hydroxyethyl cellulose, carboxymethyl cellulose, hydroxypropyl methylcellulose, and hydroxypropyl cellulose; starch and fractionated starch; agar; alginic acid and alginates; gum arabic; pullullan; agarose; carrageenan; dextrans; dextrins; fructans; inulin; mannans; xylans; arabinans; chitosans; glycogens; glucans; and synthetic biopolymers; as well as gums such as xanthan gum; guar gum; locust bean gum; gum arabic; tragacanth gum; and karaya gum; and derivatives and mixtures thereof. The preferred gelling agent herein is one that is inert to biological systems, nontoxic, simple to prepare, and not too runny or viscous, and will not destabilize the Wnt polypeptide held within it.

Preferably the polysaccharide is an etherified cellulose derivative, more preferably one that is well defined, purified, and listed in USP, *e.g.*, methylcellulose and the hydroxyalkyl cellulose derivatives, such as hydroxypropyl cellulose, hydroxyethyl cellulose, and hydroxypropyl methylcellulose. Most preferred herein is methylcellulose.

The polyethylene glycol useful for gelling is typically a mixture of low and high molecular weight PEGs to obtain the proper viscosity. For example, a mixture of a PEG of molecular weight 400-600 with one of molecular weight 1500 would be effective for this purpose when mixed in the proper ratio to obtain a paste.

The term "water soluble" as applied to the polysaccharides and PEGs is meant to include colloidal solutions and dispersions. In general, the solubility of the cellulose derivatives is determined by the degree of substitution of ether groups, and the stabilizing derivatives useful herein should have a sufficient quantity of such ether groups per anhydroglucose unit in the cellulose chain to render the derivatives water soluble. A degree of ether substitution of at least 0.35 ether groups per anhydroglucose unit is generally sufficient. Additionally, the cellulose derivatives may be in the form of alkali metal salts, for example, the Li, Na, K, or Cs salts.

If methylcellulose is employed in the gel, preferably it comprises about 2-5%, more preferably about 3%, of the gel and the Wnt polypeptide is present in an amount of about 300-1000 mg per ml of gel.

An effective amount of Wnt polypeptide to be employed therapeutically will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the patient. Accordingly, it will be

necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect. Typically, the clinician will administer the Wnt polypeptide until a dosage is reached that achieves the desired effect. A typical daily dosage for systemic treatment might range from about 1 μg/kg to up to 10 mg/kg or more, depending on the factors mentioned above. As an alternative general proposition, the Wnt polypeptide receptor is formulated and delivered to the target site or tissue at a dosage capable of establishing in the tissue a Wnt polypeptide level greater than about 0.1 ng/cc up to a maximum dose that is efficacious but not unduly toxic. This intra-tissue concentration should be maintained if possible by the administration regime, including by continuous infusion, sustained release, topical application, or injection at empirically determined frequencies. The progress of this therapy is easily monitored by conventional assays.

C. Non-Therapeutic Uses for the Wnt Polypeptide

Wnt nucleic acid is useful for the preparation of Wnt polypeptide by recombinant techniques exemplified herein which can then be used for production of anti-Wnt antibodies having various utilities described below.

The Wnt polypeptide (polypeptide or nucleic acid) can be used to induce proliferation and/or differentiation of cells *in vitro*. In particular, it is contemplated that this molecule may be used to induce proliferation of stem cell/progenitor cell populations (*e.g.* flASK cell populations obtained as described in Example 2 below). These cells which are to be grown *ex vivo* may simultaneously be exposed to other known growth factors or cytokines, such as those described herein. This results in proliferation, differentiation and/or maintenance of the cells.

In yet another aspect of the invention, the Wnt polypeptide may be used for affinity purification of Wnt receptor. Briefly, this technique involves: (a) contacting a source of Wnt receptor with an immobilized Wnt polypeptide under conditions whereby the Wnt receptor to be purified is selectively adsorbed onto the immobilized receptor; (b) washing the immobilized Wnt polypeptide and its support to remove non-adsorbed material; and (c) eluting the Wnt receptor molecules from the immobilized Wnt polypeptide to which they are adsorbed with an elution buffer. In an embodiment of affinity purification, Wnt polypeptide is covalently attaching to an inert and porous matrix (*e.g.*, agarose reacted with cyanogen bromide). Preferred is a Wnt polypeptide immunoadhesin immobilized on a protein A column. A solution containing Wnt receptor is then passed through the chromatographic material. The Wnt receptor adsorbs to the column and is subsequently released by changing the elution conditions (*e.g.* by changing pH or ionic strength).

The Wnt polypeptide may be used for competitive screening of potential agonists or antagonists for binding to the cell surface receptors. Such agonists or antagonists may constitute potential therapeutics.

A preferred technique for identifying molecules which bind to the Wnt polypeptide utilizes a chimeric polypeptide (*e.g.*, epitope tagged Wnt polypeptide or Wnt polypeptide immunoadhesin) attached to a solid phase, such as the well of an assay plate. Binding of molecules which are optionally labelled (*e.g.*, radiolabelled) to the immobilized receptor can be evaluated.

The Wnt polypeptides are also useful as molecular weight markers. To use a Wnt polypeptide as a molecular weight marker, gel filtration chromatography or SDS-PAGE, for example, will be used to separate protein(s) for which it is desired to determine their molecular weight(s) in substantially the normal way. The

Wnt polypeptide and other molecular weight markers will be used as standards to provide a range of molecular weights. For example, phosphorylase b (mw = 97,400), bovine serum albumin (mw = 68,000), ovalbumin (mw = 46,000), a Wnt polypeptide (e.g., mw = 38,000 - 42,000 depending on the coding sequence as described by *Gavin et al., supra*), trypsin inhibitor (mw = 20,100), and lysozyme (mw = 14,400) can be used as mw markers. The other molecular weight markers mentioned here can be purchased commercially from Amersham Corporation, Arlington Heights, IL. The molecular weight markers are generally labeled to facilitate detection thereof. For example, the markers may be biotinylated and following separation can be incubated with streptavidin-horseradish peroxidase so that the various markers can be detected by light detection.

The purified Wnt polypeptide, and the nucleic acid encoding it, may also be sold as reagents for mechanism studies of Wnt polypeptide and its receptor, to study the role of the Wnt polypeptide and Wnt receptor in normal growth and development, as well as abnormal growth and development, *e.g.*, in malignancies, or in diseases or disorders.

D. Wnt Polypeptide Antibody Preparation

1. Polyclonal antibodies

Potential therapeutic applications for anti-Wnt antibodies, in particular neutralizing antibodies, include the treatment of disorders, stem cell tumors and other tumors at sites of Wnt expression, including those tumors characterized by overexpressions of Wnts.

Polyclonal antibodies are generally raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, *e.g.*, keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N=C=NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining 1 mg or 1 μg of the peptide or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

2. Monoclonal antibodies

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies. For example, the monoclonal antibodies may be

made using the hybridoma method first described by Kohler *et al.*, *Nature* 256:495 (1975), or may be made by recombinant DNA methods (Cabilly *et al.*, *supra*).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 cells available from the American Type Culture Collection, Rockville, Maryland USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (*Kozbor*, J. Immunol., 133: 3001 [1984]); *Brodeur et al.*, Monoclonal Antibody Production Techniques and Applications, pp. 51-63 (Marcel Dekker, Inc., New York, [1987]).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of *Munson et al.*, *Anal. Biochem.* 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (*Goding, supra*). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown *in vivo* as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such

as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include *Skerra et al.*, Curr. Opinion in Immunol., 5: 256-262 [1993] and *Plückthun*, Immunol. Revs., 130: 151-188 [1992].

In a further embodiment, antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in *McCafferty et al.*, Nature, 348: 552-554 [1990]. *Clackson et al.*, Nature, 352: 624-628 [1991] and *Marks et al.*, J. Mol. Biol., 222: 581-597 [1991] describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (*Mark et al.*, Bio/Technology, 10: 779-783 [1992]), as well as combinatorial infection and *in vivo* recombination as a strategy for constructing very large phage libraries (*Waterhouse et al.*, Nuc. Acids. Res., 21: 2265-2266 [1993]). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (*Cabilly et al., supra*; *Morrison, et al.*, Proc. Nat. Acad. Sci. USA, 81: 6851 [1984]), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

Chimeric or hybrid antibodies also may be prepared *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide-exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate.

3. Humanized and human antibodies

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones *et al.*, *Nature* 321:522-525 (1986); *Riechmann et al.*, Nature, 332: 323-327 [1988]; *Verhoeyen et al.*, Science, 239: 1534-1536 [1988]), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (Cabilly *et al., supra*), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody (*Sims et al.*, J. Immunol., 151: 2296 [1993]; *Chothia et al.*, J. Mol. Biol., 196: 901 [1987]). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (*Carter et al.*, Proc. Natl. Acad. Sci. USA, 89: 4285 [1992]; *Presta et al.*, J. Immnol., 151: 2623 [1993]).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, *i.e.*, the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the consensus and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

Alternatively, it is now possible to produce transgenic animals (*e.g.*, mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, *e.g.*, *Jakobovits et al.*, Proc. Natl. Acad. Sci. USA, 90: 2551 [1993]; *Jakobovits et al.*, Nature, 362: 255-258 [1993]; *Bruggermann et al.*, Year in Immuno., 7: 33 [1993]. Human antibodies can also be produced in phage- display libraries (*Hoogenboom et al.*, J. Mol. Biol., 227: 381 [1991]; *Marks et al.*, J. Mol. Biol., 222: 581 [1991]).

4. Bispecific antibodies

Bispecific antibodies (BsAbs) are antibodies that have binding specificities for at least two different antigens. BsAbs can be used as tumor targeting or imaging agents and can be used to target enzymes or toxins to a cell possessing the Wnt polypeptide. Such antibodies can be derived from full length antibodies or antibody fragments (*e.g.* $F(ab')_2$ bispecific antibodies). In accordance with the present invention, the BsAb may possess one arm which binds the Wnt polypeptide and another arm which binds to a cytokine or another cytokine receptor (or a subunit thereof) such as the receptors for TPO, EPO, G-CSF, IL-4, IL-7, GH, PRL; the α or β

subunits of the IL-3, GM-CSF, IL-5, IL-6, LIF, OSM and CNTF receptors; or the α, β or γ subunits of the IL-2 receptor complex.

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein *et al.*, *Nature* 305:537-539(1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, published 13 May 1993, and in *Traunecker et al.*, EMBO J., 10: 3655-3659 [1991].

According to a different and more preferred approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690 published March 3, 1994. For further details of generating bispecific antibodies see, for example, *Suresh et al.*, Methods in Enzymology, 121: 210 [1986].

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (US Patent No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in US Patent No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. The following techniques can also be used for the production of bivalent antibody fragments

which are not necessarily bispecific. According to these techniques, Fab'-SH fragments can be recovered from *E. coli*, which can be chemically coupled to form bivalent antibodies. *Shalaby et al.*, J. Exp. Med., 175: 217-225 [1992] describe the production of a fully humanized BsAb $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling *in vitro* to form the BsAb. The BsAb thus formed was able to bind to cells overexpressing the HER2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets. See also *Rodrigues et al.*, Int. J. Cancers (Suppl.), 7: 45-50 [1992]. Various techniques for making and isolating bivalent antibody fragments directly from recombinant cell culture have also been described. For example, bivalent heterodimers have been produced using leucine zippers. *Kostelny et al.*, J. Immunol., 148(5): 1547-1553 [1992]. The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. The "diabody" technology described by *Hollinger et al.*, Proc. Natl. Acad. Sci. USA, 90: 6444-6448 [1993] has provided an alternative mechanism for making BsAb fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making BsAb fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber *et al.*, *J. Immunol.* 152:5368 (1994).

E. Articles of Manufacture

In another embodiment of the invention, an article of manufacture containing materials useful for the treatment of the conditions described above is provided. The article of manufacture comprises a container and a label. Suitable containers include, for example, bottles, vials, syringes, and test tubes. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for treating the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). The active agent in the composition is the Wnt polypeptide. The label on, or associated with, the container indicates that the composition is used for treating the condition of choice. The article of manufacture may further comprise a second container holding a cytokine for co-administration with the Wnt polypeptide. Further container(s) may be provided with the article of manufacture which may hold, for example, a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution or dextrose solution. The article of manufacture may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

F. Non-Therapeutic Uses for Antibodies to Wnts

Wnt polypeptide antibodies are also useful as affinity purification agents. In this process, the antibodies against Wnts are immobilized on a suitable support, such a Sephadex resin or filter paper, using methods well known in the art. The immobilized antibody then is contacted with a sample containing the Wnts to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the

sample except the Wnts, which is bound to the immobilized antibody. Finally, the support is washed with another suitable solvent, such as glycine buffer, pH 5.0, that will release the Wnt polypeptide from the antibody.

Wnts antibodies may also be useful in diagnostic assays for Wnt polypeptide, *e.g.*, detecting its expression in specific cells, tissues, or serum. For diagnostic applications, antibodies typically will be labeled with a detectable moiety. The detectable moiety can be any one which is capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as ^{3}H, ^{14}C, ^{32}P, ^{35}S, or ^{125}I; a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin; radioactive isotopic labels, such as, *e.g.*, ^{125}I, ^{32}P, ^{14}C, or ^{3}H; or an enzyme, such as alkaline phosphatase, beta-galactosidase, or horseradish peroxidase.

Any method known in the art for separately conjugating the polypeptide variant to the detectable moiety may be employed, including those methods described by *Hunter et al.*, Nature, 144: 945 [1962]; *David et al.*, Biochemistry, 13: 1014 [1974]; *Pain et al.*, J. Immunol. Meth, 40: 219 [1981]; and *Nygren*, J. Histochem. and Cytochem., 30: 407 [1982].

The antibodies of the present invention may be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays. *Zola, Monoclonal Antibodies: A Manual of Techniques*, pp.147-158 (CRC Press, Inc., 1987).

Competitive binding assays rely on the ability of a labeled standard to compete with the test sample analyte for binding with a limited amount of antibody. The amount of Wnt polypeptide in the test sample is inversely proportional to the amount of standard that becomes bound to the antibodies. To facilitate determining the amount of standard that becomes bound, the antibodies generally are insolubilized before or after the competition, so that the standard and analyte that are bound to the antibodies may conveniently be separated from the standard and analyte which remain unbound.

Sandwich assays involve the use of two antibodies, each capable of binding to a different immunogenic portion, or epitope, of the protein to be detected. In a sandwich assay, the test sample analyte is bound by a first antibody which is immobilized on a solid support, and thereafter a second antibody binds to the analyte, thus forming an insoluble three-part complex. See, *e.g.*, US Pat No. 4,376,110. The second antibody may itself be labeled with a detectable moiety (direct sandwich assays) or may be measured using an anti-immunoglobulin antibody that is labeled with a detectable moiety (indirect sandwich assay). For example, one type of sandwich assay is an ELISA assay, in which case the detectable moiety is an enzyme.

III. Experimental

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

The disclosures of all publications, patents and patent applications cited herein, whether *supra* or *infra*, are hereby incorporated by reference in their entirety.

EXAMPLE 1

Scr ening of *Wnt* Genes in Cells and Cell Lines

Both hematopoietic stem cell populations and stromal cell lines which support hematopoietic stem/progenitor cell growth were surveyed for *Wnt* expression by RT-PCR analysis. For these experiments, fetal liver and bone marrow hematopoietic stem/progenitor cell populations were prepared essentially as described (see, *e.g.*, Example 2A of PCT/US95/03718; and *Zeigler et al.*, Blood, 84: 2422-2430 [1994].

Briefly, day 14-15 fetal livers were made into a single cell suspension and $AA4^+$ cells were positively selected by immunoadherent panning using the $AA4^+$ antibodies purified from hybridoma supernatents as described by *Zeigler et al., supra.* Sca^+ c-kit^+ dual positive cells were recovered from the $AA4^+$ cell population by flow cytometric sorting using the Ly6 A/E phycoerythrin conjugate (Pharmingan, San Diego, CA) to recover Sca^+ cells and using fluorescein conjugated antibodies to c-kit (also from Pharmingan). $Lin^{lo}Sca^+$ bone marrow cells were recovered by magnetic bead depletion (Dynal, Inc., Great Neck, NJ; *Ploemacher et al.*, Blood, 74: 2755-2763 [1989]) of lineage-antigen expressing cells from total bone marrow and selection of $Lin^{lo}Sca^+$ cells by flow cytometric sorting using Lin cocktail antibodies from Caltag (South Francisco, CA) as described (see, Example 2A of PCT/US95/03718; and *Zeigler et al., supra.*

For the RT-PCR analysis of these cell subpopulations, PCRs were carried out with Taq polymerase (Cetus) on $AA4^+Sca^+$ cDNA or a 7-4 cell line cDNA library using each sense primer (LL1-7) with the Wnt3 antisense primer (sense primers: LL1 5' CAA GAG TGC AAA TGC CAC GGG ATG TCC GGC TCC TGC 3' (SEQ ID NO: 2); LL2 5' CAA GAG TGC AAA TGC CAC GGG GTG TCC GGC TCC TGC 3' (SEQ ID NO: 3); LL3 5' CTC AAG TGC AAA TGC CAC GGG CTA TCT GGC AGC TGT 3' (SEQ ID NO: 4); LL4 5' GTG GAG TGC AAG TGC CAC GGG GTG TCC GGC TCC TGC 3' (SEQ ID NO: 5); LL5 5' GTA GCC TGT AAG TGC CAT GGA GTG TCT GGC TCC TGT 3' (SEQ ID NO: 6); LL6 5' ACC GGG TGT AAG TGC CAT GGG CTT TCG GGT TCC TGC 3' (SEQ ID NO: 7); LL7 5' CTG GAG TGT AAG TGC CAT GGT GTG TCA GGC TCC TGT 3' (SEQ ID NO: 8); antisense primer "Wnt3" 5' GCC (C/G)CG GCC (G/A)CA (G/A)CA CAT 3' (SEQ ID NO: 9)). Additional PCRs were performed with the primer Wnt1a (5'(G/C)TG GA(A/G) TG(C/T) AA(A/G) TG(C/T) CAT 3' (SEQ ID NO: 10) and Wnt2a 5' (A/G)CA (A/G)CA CCA (A/G)TG (A/G)AA 3' (SEQ ID NO: 11). PCR products were cloned as a blunt-ended fragments into *Sma*I-linearized pGEM7 (Promega) and screened for *Wnt* sequences by hybridization with the oligonucleotides Liem1 5' GAC CTG GTG TAC 3' (SEQ ID NO: 12) or Liem2 5' TG(T/C) TG(T/C) GGC CG(G/C) GGC 3' (SEQ ID NO: 13). This analysis was confirmed using the following specific primers for *Wnt*-5a and *Wnt*-10b, using *Wnt*-3a as a negative control: *Wnt*-3a (wn3a.2, 5' CAG CCC AGG CGT CCG CGC TC3' (SEQ ID NO: 14); wn3a.3, 5' GGA ATG AAC CCT GCT CCC GT 3' (SEQ ID NO: 15)), *Wnt*-5a (wn5a1050, 5' CGC GCC CCA AGG ACC TGC CTC G 3' (SEQ ID NO: 16); wn5aR1499, 5' GCG AGC CAG TGC TCT CGT TGC G 3' (SEQ ID NO: 17)); *Wnt*-10b (W10.1, 5' AAA CCT GAA GCG GAA GTG CAA ATG C 3' (SEQ ID NO: 18); W10.3, 5' GCT CAC CTT CAT TTA CAC ACA TTG A 3' (SEQ ID NO: 19)).

These experiments detected only *Wnt*-5a and *Wnt*-10b in fetal liver $AA4^+Sca^+$ cells. Similar experiments were performed on a fetal liver stromal cell line, 7-4 (prepared as described in co-pending and coassigned PCT/US95/03718 (WO95/27062); and *Zeigler et al., supra*), where expression of only *Wnt*-5a was

detected. Thus highly enriched stem/progenitorcells and stromal cells that support their expansion were found to express *Wnts*.

These observations were extended by RT-PCR on mRNAs from the highly enriched fetal liver stem cell population $AA4^+Sca^+kit^+$ ("flASK" cells, prepared as described in co-pending and coassigned PCT/US95/03718 (WO95/27062); and *Zeigler et al., supra,* using the antibody reagents described above). *Wnt*-5a, and *Wnt*-10b, but not *Wnt*-3a, were detected in the flASK cells. Moreover, the lack of *Wnt*-3a expression in flASK cells demonstrated that, at the level of detection of the RT-PCR assays, hematopoietic stem cells express only a subset of the possible *Wnt* genes.

In additional experiments, *Wnt*-5a and *Wnt*-10b mRNAs were shown to be expressed in the stem cell populations $AA4^+$, $AA4^+Sca^+flk2^+$ and $AA4^+Sca^+flk2^-$(*Zeigler et al.,supra*). Importantly, the expression of these *Wnt* mRNAs in three different hematopoietic stem/progenitor cell subsets, each purified independently by virtue of c-kit or flk2 expression, and all three cell subsets capable of long term engraftment of lethally irradiated animals, strongly suggested a role for these ligands in the local microenvironment of the hematopoietic stem/progenitor cell. The expression of *Wnt*-5a in a fetal liver stromal cell line, 7-4, described above, and of *Wnt*-5a and *Wnt*-10b mRNAs in more mature ($AA4^-$) fetal liver hematopoietic cells further suggested (i) that much of the fetal liver hematopoietic microenvironment, as well as hematopoietic stem/progenitor cells, could potentially serve as a source of *Wnts* and (ii) that the expression of *Wnt* on these cells could allow for *Wnt*-mediated paracrine or autocrine regulation of the differentiation of these cells.

EXAMPLE 2

Analysis of *Wnts* in Hematopoiesis

A. Expansion of Hematopoietic Stem/Progenitor Cells Stimulated by Conditioned Media From *Wnt*-Transfected Cells

An *in vitro* stroma-free suspension culture system was developed to study the function of *Wnts* on highly enriched hematopoietic stem/progenitor cell populations prepared as described in Example 1. For these experiments, suspension culture of the enriched sorted cells was carried out in 24 well Costar dishes with 5000 cells/well seeded into 0.5 ml of HSC media and cultured at 37°C with 5% CO_2. HSC media contained 50% F12/50% low glucose DMEM, 10% heat-treated fetal bovine serum (Hyclone), 1 mM Glutamine, and murine kit ligand (KL) as indicated (R&D Systems). Conditioned media from 293 cells transfected with cloned *Wnt* genes was added at the time of plating. Specifically, *Wnt*-5a cDNAs were cloned from a 7-4 fetal liver stromal cell line cDNA library and *Wnt*-10b cDNAs were cloned using RT-PCR from flASK cell mRNAs. For the molecular cloning of *Wnt* cDNAs, poly A^+ RNA was prepared by the Fast Track method (Invitrogen) and cDNA was made by denaturing PolyA^+RNA and dT_{18} primers in the presence of 0.1M methyl mercuric hydroxide, followed by quenching with 20 mM beta-mercaptoethanol and extension in 20 µl total with Superscript reverse transcriptase as recommended (Gibco).

A *Wnt*-5a PCR fragment was used to screen a cDNA library made from the 7-4 cell line and ligated into the pSPORT-1 vector (Gibco). The *Wnt*-5a coding region was sequenced from one clone, Wnt5a.13.pSPORT-1, and the predicted amino acids matched those previously reported (*Gavin et al.*, Genes

and Dev., 4: 2319-2332 [1990]) except for (H207>Y). A *Wnt*-10b cDNA was cloned by RT-PCR from flASK cells using primers wn10b.5ri (5'GGA ATT CCG GGC TTC GAC ATG CTG GAG GA 3' (SEQ ID NO: 20)) and wn10b.3kpn (5' GGG GTA CCC CAG GCT CAC CTT CAT TTA CAC A 3' (SEQ ID NO: 21)), and cloned into pGEM7. The predicted coding sequence matched exactly to that reported elsewhere (*Lee* et al., Proc. Natl. Acad. Sci. USA, 92: 2268-2272 [1995]).

For overexpression of murine *Wnts* in mammalian cells and the preparation of conditioned media (CM) from the cells containing *Wnt* gene products, the *Wnt*-1, *Wnt*-5a, and *Wnt*-10b cDNAs thus obtained were cloned into as EcoRI/Hind III fragments into an expression vector pRK5tkneo (*Holmes*, Science, 253: 1278-1280 [1991]). For production of CM, 293 cells were transfected by the calcium phosphate method (*Gorman*, DNA Cloning: A Practical Approach, IRL, Washington, D.C. [1985]), media was collected after at least 48 hours, centrifuged at 3,000 x g, and sterile filtered. In most experiments using CM, the CM was added to 5-10% final concentration. In preliminary experiments, most of the known cytokines failed to support the survival of flASK cells in suspension cultures when added as single factors in the presence of 10% fetal bovine serum (see, *e.g.*, *Bodine et al.*, Blood, 78: 914-920 [1991]). The addition of KL at 100 ng/ml, however, provided a potent stimulus for cell survival and proliferation of granulocytic progenitors (*Anderson* et al., Cell, 63: 235-24_ [1990]; *Zsebo et al.*, Cell, 63: 195-201 [1990]. Interestingly, control 293 CM provided an approximately 2-fold stimulatory activity when added to suspension cultures with KL. The addition of conditioned media (CM) from 293 cells transfected with a *Wnt*-5a cDNA evoked a 2-3 fold greater expansion than control CM.

The presence of Wnt-5a protein in the CM from transfected cells was confirmed by immunoprecipitations and Western blotting. For these experiments, a chimeric *Wnt*-5a gene was made encoding the first 55 amino acids (AA) of herpes simplex virus glycoprotein D followed by *Wnt*-5a AA 38-379 in gDCT-1pRK5b (*Pennica et al.*, Proc. Natl. Acad. Sci. USA, 92: 1142-1146 [1995]). The control vector used in experiments involving gDWnt5a was made from gDCT-1pRK5b by excising the CT-1 cDNA as a *XhoI-XbaI* fragment, filling in the overhanging nucleotides, and closing the gDpRK5b vector with T4 DNA ligase (Collaborative Research). The resulting construct was expected to encode the first 54 AA of gD followed by D stop. A fragment encoding six histidine residues was appended in-frame to the carboxy terminus of *Wnt*-5a in the gDpRK5b vector by PCR.

For immunoprecipitation experiments for the analysis of *Wnt* expression by transfected cells, 293 cells transfected with gDpRK5b or gDWnt5apRK5b were labeled in 100 μCi [^{35}S]methionine/[^{35}S]cysteine (Amersham), lysed, and proteins were precipitated with the 5B6 mAb plus protein A Sepharose (Pharmacia). The precipitates were washed, electrophoresed, and fluorographed according to standard methods.

For comparison, the potent cytokines IL-3 and GM-CSF added as single factors to cultures with KL evoked a 1.5-2 fold greater expansion than controls. Further experiments revealed that titration of KL to 25 ng/ml provided a lower expansion in cell number, but the cultures displayed a reduced level of granulocytic proliferation/differentiation. Addition of Wnt-1, Wnt-5a, and Wnt-10b CM to the suspension cultures of flASK cells plus 25 ng/ml KL stimulated cell proliferation 7, 8, and 11-fold (respectively) over that of control CM after 7 days in culture as shown in Figure 1. A synergistic effect between KL and Wnts was evident since Wnt CM alone triggered very little cell survival/proliferation. Thus far, Wnt-1 expression has not been detected in the

hematopoietic system, however, Wnt-1 CM was active in this assay. These results demonstrate the potential for distinct signaling pathways from several Wnt ligands leading to a similar response of hematopoietic cells or the presence of a common Wnt receptor in hematopoietic stem/progenitor cells.

B. Maintenance of the Blast Cell Phenotype and Foci Formation of Hematop ietic Progenitor Cells Enhanced by Wnts

In mice, Wnts are essential for the development of several primitive cell types and in *Xenopus* they are thought to be involved in cell fate determination. The role of Wnts was evaluated on the differentiation potential of pluripotent hematopoietic stem/progenitor cells by examining the morphology of cultured cells in cytostained preparations. For the cytospin analysis, cells were grown in suspension culture as described above, spun onto a glass slide and then stained with Wright Geimsa to reveal hematopoietic cell types and morphology. For these experiments, cytospin analysis of flASK cells was performed with cytospin preparations of (A) flask cells immediately after cell sorting, (B) flASK cells after suspension culture in control CM plus 25 ng/ml KL, and (C) flASK cells after suspension culture in Wnt-5a CM plus KL. Although addition of KL to the suspension cultures was essential, even in reduced concentrations, the effect of KL alone was to promote the differentiation and proliferation of granulocytic cells from HSCs compared to freshly isolated flASK cells. However, cells cultured in Wnt-5a or Wnt-10b CM or partially purified recombinant Wnt-5a (see Section C below) generally gave rise to a greater diversity of cell lineages with less commitment towards granulocytic lineages. Myeloid cells (macrophages and neutrophils), megakaryocytes, and early erythroid cells, were observed by cytospin analysis of suspension cultures after treatment with Wnt-5a CM. Notably, the ratio of primitive blasts to differentiated mononuclear cells was elevated over 4-fold (29% ± 3.4 compared to 7% ± 0.8 for the control) in cultures with recombinant Wnt-5a. The effect of Wnts plus KL was to promote extensive cell expansion, that is the net increase in cell number from the initial HSC inoculum, while also maintaining a 4 fold greater proportion of cells with a primitive blast cell morphology.

Wnts have been implicated in the regulation of cell adhesion systems, and it has been proposed that cell-cell interactions may be important in cell fate determination (*Hinck* et al., J. Cell. Biol., 124:729-741 [1994]; *Peiffer*, Development Suppl., 163-176 [1993]). During the first 4-5 days of suspension culture in Wnt CM, a dramatic increase in the number of loosely adherent cell aggregates or adherent hematopoietic 'foci' were observed. To analyze the spatial organization and morphology of these foci, flASK cells in HSC media with 25 ng/ml murine KL were plated onto Lab-Tek glass chamber slides (NUNC, Naperville, IL) coated with 50 mg/ml of human plasma fibronectin (Gibco), cultured for 4-5 days in control CM plus 25 ng/ml KL, and (E) *Wnt*-5a CM (gDWnt5a) plus 25ng/ml KL, and then cytostained *in situ* to preserve the intercellular organization of the foci. Fibronectin was chosen as an adhesive substrate in this assay since it can mediate adhesion of CFU-S progenitors *in vitro* (*Williams et al.*, Nature, 352: 438-441 [1991]). After 3-5 days in culture, clusters of 5 to more than 30 blasts with low cytoplasm to nucleus ratios were typically found in contact with one or more underlying adherent myeloid cells when the control culture in preparation D was compared with Wnt-5a treated cells in preparation E). The formation of these blast cell f ci was dramatically enhanced in response to Wnt CM and suggested a role for Wnts in cell expansion via the regulation of cellular interactions.

In light of the enhanced proliferation and cell-cell adhesion of cells cultured in Wnt CM, the lineage phenotypes and adhesion systems of the cultured cells were analyzed by flow cytometry. For cell analysis and sorting experiments, phycoerythrin-conjugated antibodies (Ly6A/E, TER-119, CD14), fluorescein-conjugated antibodies (c-kit, CD13, CD31, CD44, CD45, CD49d, CD49e, GR1, VCAM-1, ICAM-1, L-selectin), CD11a, and CD29 antibodies were purchased from Pharmingen; phycoerythrin-conjugated Mac-1, fluorescein-conjugated antibodies (CD4, CD8a, and B220), and all secondary and Lin cocktail antibodies were purchased from Caltag.

The flASK cells were cultured in gDWnt5a CM (Wnt CM) or gD CM (control CM) for 7 days and scored for the expression of cell surface antigens. The expression of antigens found on mature hematopoietic cells (CD4, CD8a, CD13, CD14, B220, VCAM-1, and integrin beta-7) was low or negative on freshly sorted flASK cells and remained at similar levels after culture. Little or no change was observed in either condition for expression of CD11a, CD29, CD31, CD44, CD45, CD49d, CD49e, GR1, L-selectin, ICAM-1. Cultures supplied with Wnt-5a CM did, however, have an increase in the number of cells that were Sca^+ (154±22.6%), $c\text{-}kit^+$ (158±36%), Sca^+ $c\text{-}kit^+$ (131±9.4%), or $Ter119^+$ (237±25.8%). These cell surface antigen profiles compared well with the cytospin analysis and strengthened the finding that Wnts plus KL promote the maintenance of a greater proportion of primitive blast cells than KL alone during the *ex vivo* culture of HSCs.

C. Requirement of Secreted *Wnt* Protein in Wnt-Conditioned Media for Proliferation of Hematopoietic Stem/Progenitor Cells

Several approaches were taken to distinguish a direct role of *Wnts* on HSCs from the alternative that Wnts stimulate the production of other growth factors in the transfected cells. Antibody depletion experiments were carried out to confirm that the proliferation was mediated by secreted *Wnt* proteins present in the media. An epitope-tag was engineered onto Wnt-5a to enable depletion with readily available antibody reagents. Chimeric proteins were constructed as described in Section A above that encoded the signal sequence and an N-terminal domain of the herpes virus glycoprotein D (gD) followed by Wnt-5a (gDWnt5a, $gDWnt5aHis_6$. Transfection of the gDWnt5a construct into 293 cells directed the expression of a 47-49kD polypeptide which was specifically immunoprecipitated from lysates by a mAb (5B6) recognizing an N-terminal epitope of gD. Immunodepletion experiments were conducted to remove the Wnt protein produced by the 293 transfected cells (gD*Wnt*5apRK5b or control gDpRK5b) from the CM with the mAb5B6. Incubation of $gDWnt5aHis_6$ CM in mAb 5B6 bound to protein A or coupled to controlled-pore glass (5B6-CPG) reduced cell expansion to control values. Collectively, these data indicate that secreted Wnts mediated the observed cell expansions *in vitro*. To test whether Wnts could directly stimulate cell proliferation, we evaluated the activity of partially-purified Wnt-5a protein in the suspension culture assay. Preliminary experiments showed that cell extracts provided a richer source of Wnt-5a protein than CM. For the purification of $gD.Wnt5a.His_6$, stable lines of CHOdp12 cells ($DHFR^-$ cells, see, *e.g.*, *Bennett et al.*, J. Biol. Chem., 766: 23060 [1991]) that had been transfected with a $DHFR^+$ plasmid $gD.Wnt5a.His_6pSVi.del.d$ were selected and maintained in glutathione-S-transferase (GHT)-free media. Extracts were made from $gD.Wnt5a.His_6$ CHO cells as follows. Cells were lysed in 50mM Triethanolamine, 100mM NaCl, 0.4% SDS, 1% PMSF, and 2% Triton X-100. Purification of $gD.Wnt5a.His_6$ from the lysate was accomplished by binding of the gD epitope to 5B6-CPG, extensive washing in PBS, and

acid elution. The eluate was neutralized, dialyzed against PBS, and refolded in 8M urea. The refolded Wnt-5a protein was diluted in HSC media so that concentration of urea was less than 60mM Wnt-5a in the stem cell suspension culture assay.

Gel analysis revealed that the protein migrated as a 47-49kD monomer under reducing conditions. When added to the suspension culture assay, recombinant Wnt-5a protein was found to stimulate cell expansion by 5-fold at approximately 40-80 ng/ml or 1-2 nM.

D. Stimulation of Total CFC Expansion of Hematopoietic Progenitor Cells by Wnt Protein

An important measure of hematopoietic progenitor cells is the ability to form colonies in semi-solid media in response to lineage-specific cytokines and multilineage colony-stimulating factors. Since the addition of Wnts plus KL to flASK cell cultures increased the proportion of cells with a primitive morphology and cell surface phenotype, experiments were performed to analyze whether the number of highly proliferative colony-forming cells had increased as well. The frequency of colony forming cells (CFCs) in flASK cell cultures was examined by measuring colony formation of cells replated into myeloid methylcellulose containing a combination of cytokines (KL, IL-3, IL-6, and Epo) and Wnt CM or control CM. For the colony assay experiments, methylcellulose cultures were initiated in 35 mM plates with 1000 cells in 1.0 ml complete myeloid methylcellulose (Stem Cell Technologies, Inc.) or in B-cell conditions consisting of base methylcellulose containing 50 ng/ml murine KL and 50 ng/ml murine IL-7 (R&D Systems). Conditioned media was added at the time of plating. Plates were read at day 12 after plating. After suspension culture, the total CFCs derived from 1000 cells in the initial culture inoculum was 3-fold greater in Wnt-5a CM than control CM. Moreover, when cells were harvested from day 12 myeloid methylcellulose cultures and replated, the cumulative expansion of cells brought forth by Wnt-5a CM was over 235 fold greater than for control CM, largely due to the inability of cells grown in control CM to efficiently replate. These results provide compelling functional evidence that secreted Wnts enhance the survival/proliferation of multipotent hematopoietic progenitors in suspension culture and can directly stimulate the expansion of primitive, highly proliferative colony forming cells.

Colony formation of freshly isolated fetal liver $AA4^+$ Sca^+ cells in Wnt-5a CM was also tested. Wnt-5a CM stimulated colony formation approximately 3-fold in myeloid methylcellulose. In B-cell conditions, Wnt-5a CM stimulated colony formation 4-fold. Colony formation was enhanced 2-fold for bone marrow Lin^{lo} Sca+ cells in myeloid and B-cell methylcellulose. Overall, these data show that Wnt CM enhances colony formation by highly enriched fetal liver and bone marrow HSCs in conditions that detect myeloid or B-cell progenitors.

E. Expansion of Hematopoietic Stem/Progenitor Cells After Transduction with a Retrovirus Bearing a Wnt Protein

To further examine the direct effects of *Wnt* expression on HSCs, a representative Wnt protein product, Wnt-5a, was introduced via retroviral transduction. A Rous sarcoma virus-based bicistronic LNL6 vector was constructed so that *Wnt*-5a was placed 3' to the gag gene and LacZ was downstream of the encephalomyocarditis virus internal ribosome entry site. Specifically, for the viral construction and transduction experiments, *Wnt*5a.13.pSPORT-1 was digested with *Eco*RI/*Bam*HI, the insert was blunted with T4 DNA Polymerase (U.S. Biochemical), and cloned into blunted *Bgl*II/*Bam*HI sites of the pLNL6 vector (*Ghattas et al.*, Mol. Cell. Biol., 11: 5848-5859 [1991]). *Wnt*5a/LNL6 or the parental LNL6 vector were transfected by a calcium phosphate

method into BOSC 23 cells (*Pear et al.*, Proc. Natl. Acad. Sci. USA, 90: 8392-8396 [1993]). Viral supernatants from the transfected BOSC cells were collected after 48-72 hours and stored at -20°C and used to transduce flASK cells. Transductions of the flASK cells were carried out at 100,000 cells per ml for 48 hours in viral supernatants supplemented with the murine cytokines IL-3 (25 ng/ml), IL-6 (50ng/ml), and KL (10ng/ml). Transduction efficiency of cells giving rise to methylcellulose colonies was assayed by PCR analysis essentially as described in *Gerard et al.*, Human Gene Therapy, 7: 343-354 [1996]. Expression of the biscistronic mRNA in transduced flASK cells was confirmed by measuring LacZ activity using the FACS-Gal method (*Fiering et al.*, Cytometry, 12: 291-301 [1991]). The added cytokines IL-3, IL-6, and KL increased the transduction efficiency, which was estimated to be approximately 20% by FACS-Gal analysis 48 hours post-transduction. A potential early-acting effect of *Wnt*-5a on cell survival/proliferation was measured by counting cell numbers 48 hours after transduction. Wnt5a/LNL6-transduced cells expanded by almost 2-fold (Figure 2A). Notably, this cell survival/proliferation was impressive in that an additional benefit was observed over the potent effects of the early-acting cytokines IL-3, IL-6, and KL. Culture of the Wnt5a/LNL6-transduced cells for 7 days revealed extensive proliferation compared to that of control cells (Figure 2B). Cells from 2 day transductions were also replated into myeloid methylcellulose. Transduction with Wnt5a/LNL6 stimulated a 3-fold greater expansion of CFCs than the control vector (Figure 2C). The efficiency of CFC transduction was estimated to be 14-47% by PCR analysis of colonies plucked from the methylcellulose cultures.

SEQUENCE LISTING

(1) GENERAL INFORMATION:
(i) APPLICANT: Genentech, Inc.
(ii) TITLE OF INVENTION: USES FOR WNT POLYPEPTIDES
(iii) NUMBER OF SEQUENCES: 21

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Genentech, Inc.
(B) STREET: 460 Point San Bruno Blvd
(C) CITY: South San Francisco
(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 94080

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: 3.5 inch, 1.44 Mb floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: WinPatin (Genentech)

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/696566
(B) FILING DATE: 16-AUG-1996

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Svoboda, Craig G.
(B) REGISTRATION NUMBER: 39,044
(C) REFERENCE/DOCKET NUMBER: P1034PCT

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 415/225-1489
(B) TELEFAX: 415/952-9881
(C) TELEX: 910/371-7168

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Asp Lys Thr His Thr Cys Pro Pro Cys Pro
1 5 10

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs

(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CAAGAGTGCA AATGCCACGG GATGTCCGGC TCCTGC 36

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CAAGAGTGCA AATGCCACGG GGTGTCCGGC TCCTGC 36

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CTCAAGTGCA AATGCCACGG GCTATCTGGC AGCTGT 36

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GTGGAGTGCA AGTGCCACGG GGTGTCCGGC TCCTGC 36

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GTAGCCTGTA AGTGCCATGG AGTGTCTGGC TCCTGT 36

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ACCGGGTGTA AGTGCCATGG GCTTTCGGGT TCCTGC 36

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CTGGAGTGTA AGTGCCATGG TGTGTCAGGC TCCTGT 36

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GCCSCGGCCR CARCACAT 18

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

STGGARTGYA ARTGYCAT 18

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

RCARCACCAR TGRAA 15

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GACCTGGTGT AC 12

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TGYTGYGGCC GSGGC 15

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CAGCCCAGGC GTCCGCGCTC 20

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs

(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GGAATGAACC CTGCTCCCGT 20

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CGCGCCCCAA GGACCTGCCT CG 22

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GCGAGCCAGT GCTCTCGTTG CG 22

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

AAACCTGAAG CGGAAGTGCA AATGC 25

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GCTCACCTTC ATTTACACAC ATTGA 25

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GGAATTCCGG GCTTCGACAT GCTGGAGGA 29

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GGGGTACCCC AGGCTCACCT TCATTTACAC A 31

WHAT IS CLAIMED IS:

1. A method for enhancing proliferation, differentiation or maintenance of a hematopoietic stem/progenitor cell comprising exposing the cell to an amount of *Wnt* polypeptide which is effective for enhancing proliferation, differentiation or maintenance of the cell.

2. The method of claim 1 wherein the cell is a $CD34^+$ cell.

3. The method of claim 1 wherein the cell is a $AA4^+$ cell.

4. The method of claim 1 wherein the cell is a flASK cell.

5. The method of claim 1 which enhances proliferation of the cell.

6. The method of claim 1 which enhances differentiation of the cell.

7. The method of claim 1 which enhances maintenance of the cell.

8. The method of claim 1 further comprising exposing the cell to a further cytokine.

9. The method of claim 8 wherein the further cytokine is a lineage-specific cytokine.

10. The method of claim 8 wherein the further cytokine is selected from the group consisting of thrombopoietin (TPO); erythropoietin (EPO); macrophage-colony stimulating factor (M-CSF); granulocyte-macrophage-CSF (GM-CSF); granulocyte-CSF (G-CSF); interleukin-1 (IL-1); IL-1α; IL-2; IL-3; IL-4; IL-5; IL-6; IL-7; IL-8; IL-9; IL-11; IL10; IL-12; leukemia inhibitory factor (LIF) and kit ligand (KL).

11. The method of claim 1 wherein the cell is in cell culture.

12. The method of claim 1 wherein the cell is present in a mammal.

13. The method of claim 12 wherein the mammal is a human.

14. The method of claim 12 wherein the mammal is suffering from, or is expected to suffer from, decreased blood cell levels.

15. The method of claim 14 wherein the decreased blood cell levels are caused by chemotherapy, radiation therapy, or bone marrow transplantation therapy.

16. A method for repopulating blood cells in a mammal comprising administering to the mammal a therapeutically effective amount of a *Wnt* polypeptide.

17. The method of claim 16 wherein the blood cells are erythroid cells.

18. The method of claim 16 wherein the blood cells are myeloid cells.

19. The method of claim 16 wherein the blood cells are lymphoid cells.

20. The method of claim 16 comprising administering a further cytokine to the mammal in an amount which leads to a synergistic repopulation of the blood cells in the mammal.

21. The method of claim 20 wherein the further cytokine is erythropoietin (EPO); granulocyte-macrophage-colony stimulating factor (GM-CSF); kit ligand (KL); or interleukin-3 (IL-3).

22. A pharmaceutical composition comprising *Wnt* polypeptide, a further cytokine, and a physiologically acceptable carrier.

23. An article of manufacture, comprising:

a container;

a label on the container; and

a composition comprising an active agent contained within the container; wherein the composition is effective for repopulating blood cells in a mammal, the label on the container indicates that the composition can be used for repopulating blood cells in a mammal and the active agent in the composition is a *Wnt* polypeptide.

24. The article of manufacture of claim 23 comprising a further container which holds a further cytokine.

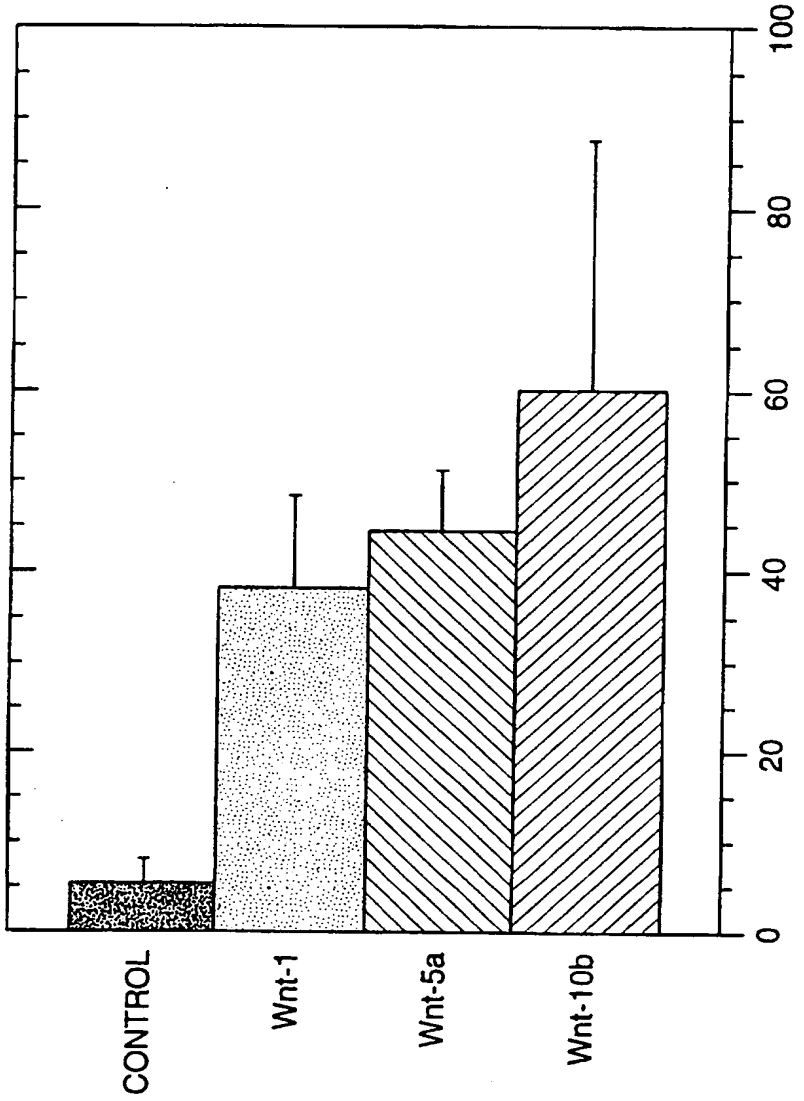


FIG._1

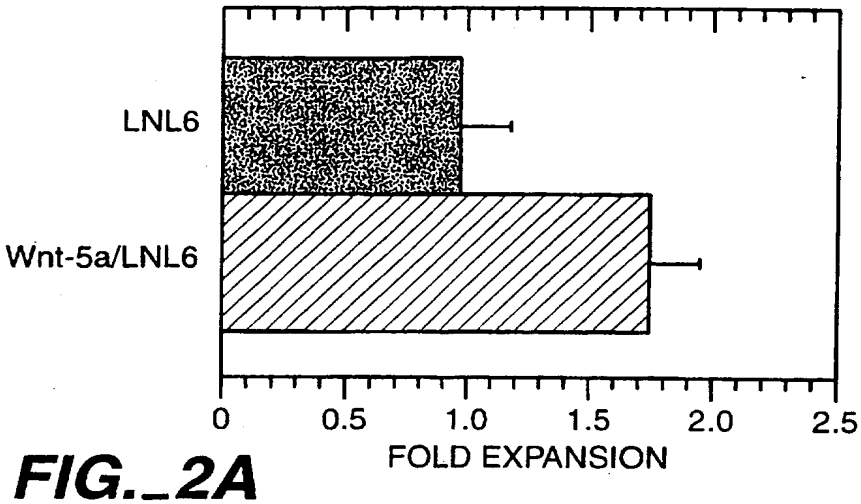


FIG._2A

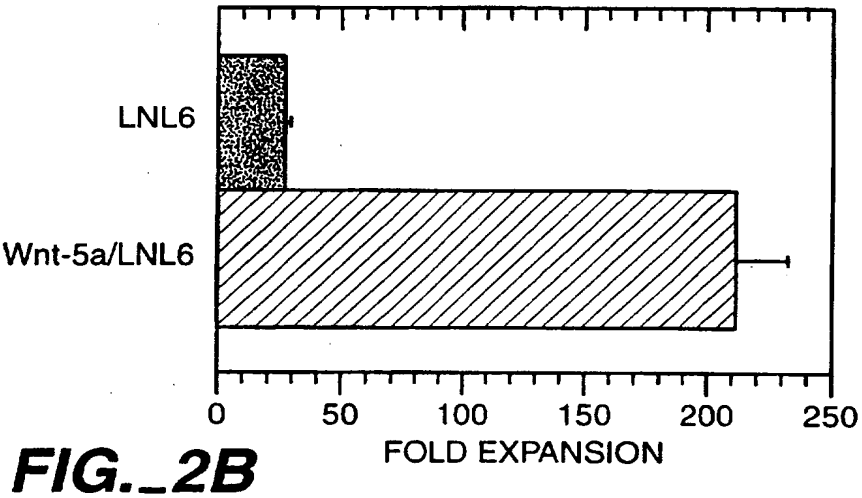


FIG._2B

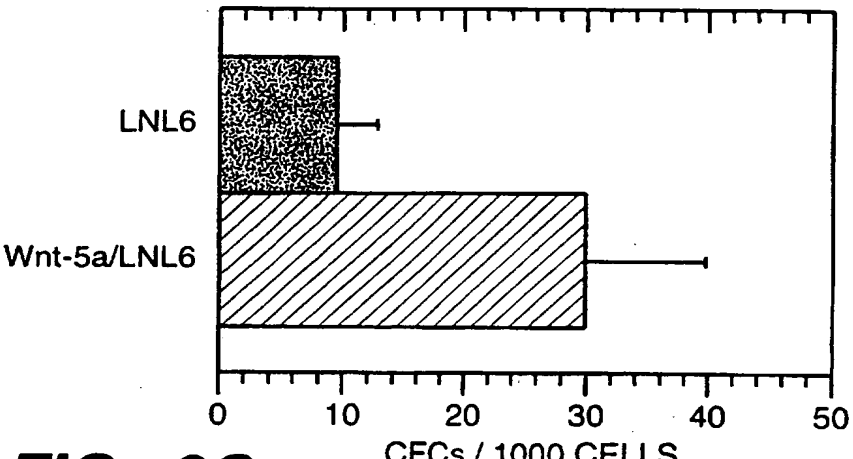


FIG._2C

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A61K 38/18

A3

(11) International Publication Number: WO 98/06747

(43) International Publication Date: 19 February 1998 (19.02.98)

(21) International Application Number: PCT/US97/13910

(22) International Filing Date: 7 August 1997 (07.08.97)

(30) Priority Data:
08/696,566 16 August 1996 (16.08.96) US

(71) Applicant: GENENTECH, INC. [US/US]; 1 DNA Way, South San Francisco, CA 94080-4990 (US).

(72) Inventors: MATTHEWS, William; 560 Summit Springs Road, Woodside, CA 94062 (US). AUSTIN, Timothy, W.; 1010 Peebles Avenue, Morgan Hill, CA 95037 (US).

(74) Agents: SVOBODA, Craig, G. et al.; Genentech, Inc., 1 DNA Way, South San Francisco, CA 94080-4990 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

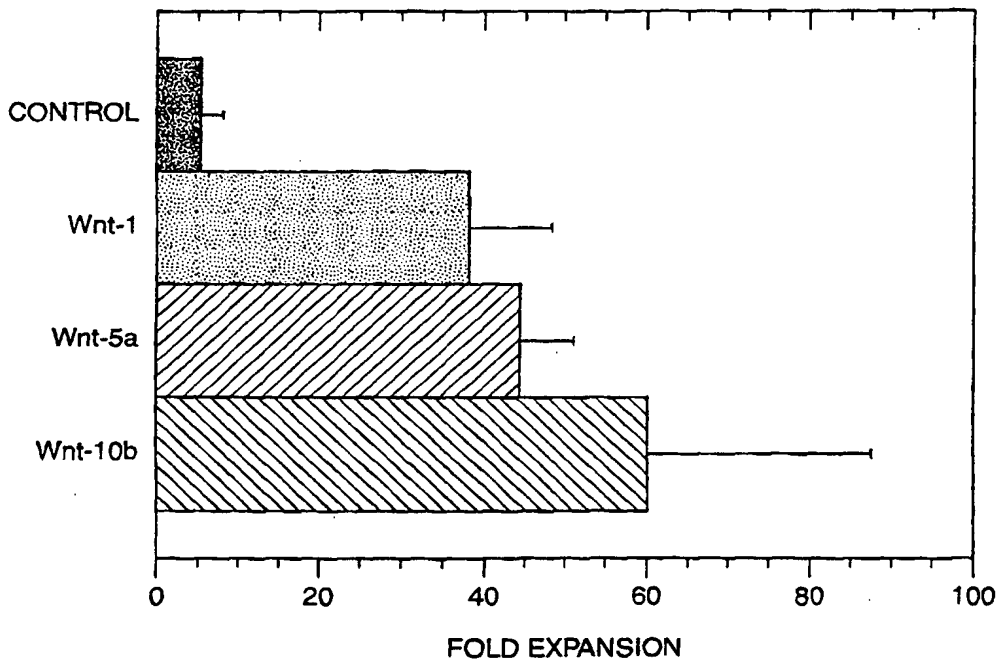
Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the international search report:
7 May 1998 (07.05.98)

(54) Title: USES FOR WNT POLYPEPTIDES



(57) Abstract

Uses for *Wnt* polypeptides in hematopoiesis are disclosed. In particular, *in vitro* and *in vivo* methods for enhancing proliferation, differentiation or maintenance of a hematopoietic stem/progenitor cell using a *Wnt* polypeptide, and optionally another cytokine, are described.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

INTERNATIONAL SEARCH REPORT

Int. Application No
PCT/US 97/13910

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	TIMOTHY W. AUSTIN ET AL.: "A ROLE FOR THE WNT GENE FAMILY IN HEMATOPOIESIS:EXPANSION OF MULTILINEAGE PROGENITOR CELLS." BLOOD, vol. 89, no. 10, 15 May 1997, pages 3624-3635, XP002058232 see the whole document ---	1-24
X,P	D. J. VAN DEN BERG ET AL.: "A ROLE FOR THE MEMBERS OF THE WNT GENE FAMILY IN HUMAN HEMATOPOIESIS." BLOOD, vol. 88, no. 10, 15 November 1996, page 141A XP002058233 see paragraph 554 --- -/--	1-24

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
9 March 1998	25. 03. 1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Rempp, G

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/13910

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 95 17416 A (MERCK & CO INC ;RODAN GIDEON A (US); RUTLEDGE SU JANE (US); SCHMID) 29 June 1995 cited in the application	
A	WO 94 02157 A (INDIANA UNIVERSITY FOUNDATION) 3 February 1994 cited in the application	

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 97/13910

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

Claims Nos.: 16-21

because they relate to subject matter not required to be searched by this Authority, namely:

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

Remark : Although claims 16-21
are directed to a method of treatment of
the human/animal body , the search has been carried out and based on the
alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte Application No
PCT/US 97/13910

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9517416 A	29-06-95	EP 0736034 A	09-10-96
		JP 9507750 T	12-08-97
WO 9402157 A	03-02-94	US 5672346 A	30-09-97
		AU 4788093 A	14-02-94
		CA 2141207 A	03-02-94
		EP 0658114 A	21-06-95

Development 125, 4225-4234 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV2327

Wnt-4 is a mesenchymal signal for epithelial transformation of metanephric mesenchyme in the developing kidney

Andreas Kispert[1,2,*], Seppo Vainio[1,‡] and Andrew P. McMahon[1,*]

[1]Department of Molecular and Cellular Biology, Harvard University, 16 Divinity Avenue, Cambridge, MA 02138, USA
[2]Max-Planck-Institut für Immunbiologie, Abteilung für Molekulare Embryologie, Stübeweg 51, 79108 Freiburg, Germany
*Authors for correspondence (e-mail: kispert@immunbio.mpg.de; amcmahon@biosun.harvard.edu)
‡Present address: University of Oulu, Department of Biochemistry, Linnanmaa, Oulu 90570, Finland

Accepted 21 August; published on WWW 30 September 1998

SUMMARY

Development of the mammalian kidney is initiated by ingrowth of the ureteric bud into the metanephric blastema. In response to signal(s) from the ureter, mesenchymal cells condense, aggregate into pretubular clusters, and undergo epithelialisation to form simple epithelial tubules. Subsequent morphogenesis and differentiation of the tubular epithelium lead to the establishment of a functional nephron.

Here we demonstrate that Wnt-4, a secreted glycoprotein which is required for tubule formation, is sufficient to trigger tubulogenesis in isolated metanephric mesenchyme, whereas Wnt-11 which is expressed in the tip of the growing ureter is not. Wnt-4 signaling depends on cell contact and sulphated glycosaminoglycans and is only required for triggering tubulogenesis but not for later events. The Wnt-4 signal can be replaced by other members of the *Wnt* gene family including Wnt-1, Wnt-3a, Wnt-7a and Wnt-7b. Further, dorsal spinal cord, which has been thought to mimic ureteric signaling in tubule induction induces *Wnt-4* mutant as well as wild-type mesenchyme suggesting that spinal cord derived signal(s) most likely act by mimicking the normal mesenchymal action of Wnt-4. These results lend additional support to the notion that Wnt-4 is a key auto-regulator of the mesenchymal to epithelial transformation that underpins nephrogenesis adding another level of complexity in the hierarchy of molecular events mediating tubulogenesis.

Key words: Wnt, Wnt-4, Induction, Tubulogenesis, Kidney development, Metanephros

INTRODUCTION

The development of vertebrate organs requires intricate cell and tissue interactions to assure the concerted program of cell growth, differentiation and morphogenesis. The mammalian kidney, mainly of mouse and rat, has long been studied as a model system to reveal both the embryological principles and more recently the molecular control of vertebrate organ formation (for reviews see Saxen, 1987; Lechner and Dressler, 1997; Vainio and Muller, 1997).

Mouse renal development is characterised by the continuous interaction of epithelial and mesenchymal compartments both of which derive from the intermediate mesenchyme. These are the nephric duct and its derivative the ureter, and the nephrogenic mesenchyme which lies adjacent to these ducts. As a consequence of these interactions three embryonic kidneys are laid down from anterior to posterior in time and space. While the initial organ, the pronephros is only a very transient structure established at 8-8.5 days post coitum (d p.c.), the mesonephros extends by posterior elongation of the nephric duct and subsequent tubule induction in the adjacent mesonephrogenic mesenchyme between 9 and 11 d p.c. Although forming elaborate tubules, the mesonephros of the male never becomes a functional organ but contributes to the ductal network of the rete testis.

Metanephric development is initiated when a bud emerges from the nephric duct at the level of the hind limbs around 10.5 d p.c. The ureteric duct subsequently invades the metanephric blastema which lies at the posterior end of the intermediate mesoderm.

In a process repeated many times, mesenchymal cells condense around the tip of the ureter, i.e. bud, aggregate, epithelialise and undergo morphogenetic movements and cellular differentiation programs to form a major part of the nephron the functional unit of the vertebrate kidney. The ureter continues to grow and to branch forming the collecting duct system of the mature organ. 7-10 days post partum nephron formation ceases most likely as the mesenchymal stem cells in the periphery of the kidney are exhausted.

In order to achieve the complex architecture of the mature kidney, the morphogenetic and cellular differentiation programs of both the nephric duct and the mesenchymal derivatives have to be highly integrated making it very likely that multiple signaling systems between and within the two compartments are operative.

Classical kidney organ culture experiments have primarily

focused on the signals exchanged between mesenchyme and ureter upon their initial contact. Separation and recombination experiments have shown that isolated metanephric mesenchyme undergoes apoptosis unless provided with a permissive stimulus which leads to epithelial tubule formation (Grobstein, 1953; Saxen, 1987). Similarly, ureter tissue degenerates upon separation from metanephric mesenchyme, and undergoes limited or altered branching morphogenesis when recombined with heterologous mesenchymal tissues suggesting that metanephric mesenchyme secretes signals essential for ureter survival and correct branching morphogenesis (Saxen, 1987; Kispert et al., 1996; Sainio et al., 1997).

A search for heterologous tissues that may be a more convenient source of a factor capable of replicating ureteric signaling has identified the dorsal spinal cord as a potent inducer of tubulogenesis. Indeed, most of our understanding of cell interactions in kidney development comes from the application of spinal cord derived signals to isolated metanephric mesenchyme in culture. These studies have demonstrated that induction appears to be cell contact dependent, requires approximately 24 hours of contact between spinal cord and mesenchyme, can be blocked by metabolic inhibitors, and cannot be transferred from induced to uninduced mesenchyme (homeogenetic induction) (Grobstein, 1953; Saxen, 1987, and references therein).

It is important to note that while this assay has generally been thought to provide information about ureteric-like inductive signals there is no evidence that this is the case. For example, induction of tubules may require the action of several signals, some from the ureter others from the mesenchyme. Supplying any of these may be sufficient to trigger tubule formation.

While recently progress has been made in identifying mesenchymally derived signaling molecules required for ureter proliferation and branching morphogenesis (see Sariola and Sainio, 1997, for review) the nature of ureteric signals has remained elusive. Several growth factors have been discussed as potential inducers (Hammerman; 1995) but none of them has conclusively been shown to be required and sufficient for tubule induction. A combination of FGF2 and a pituitary extract can induce tubulogenesis, suggesting that tubule induction is a multi-step process mediated by soluble and possibly insoluble factors (Perantoni, 1991; Perantoni et al., 1995).

Several findings have implicated members of the Wnt family of secreted glycoproteins in signaling processes operating during metanephric development. *Wnt-11* is expressed in the tips of the growing ureter where tubule inducing activity is thought to arise (Kispert et al., 1996). In contrast, *Wnt-4* is expressed in pretubular mesenchyme cells shortly before they aggregate and transform to simple epithelial tubules. Loss of function studies indicate that *Wnt-4* is required for tubule formation (Stark et al., 1994). Finally, *Wnt-7b* is expressed somewhat later in the collecting duct epithelium which derives from the ureteric duct (Kispert et al., 1996). NIH3T3 cells stably expressing *Wnt-1*, which is not expressed in the kidney but is expressed in the dorsal spinal cord, are able to induce tubulogenesis in isolated rat metanephric mesenchyme (Herzlinger et al., 1994). This suggests that a member of the Wnt family may normally participate in tubule induction. Thus, induction in culture by the spinal cord might mimic ureteric signaling by Wnt-11 *or* mesenchymal signaling by Wnt-4.

Here we describe a number of experiments in which we investigate the role of *Wnt-11*, *Wnt-4* and a number of other family members in tubule induction. From these results we conclude that Wnt-4, but not Wnt-11, is able to induce tubule formation, and that spinal cord mediated tubulogenesis may reflect the normal mesenchymal function of Wnt-4 rather than that of a ureteric bud derived signal.

MATERIALS AND METHODS

Mice

Wnt-4 heterozygotes were derived and genotyped as described previously (Stark et al., 1994). Embryos for kidney dissections were derived from matings of Swiss Webster (SW) wild-type animals or *Wnt-4* heterozygotes. For timed pregnancies plugs were checked in the morning after mating, noon was taken as 0.5 days post coitum (d p.c.).

Cell lines

Cell lines stably expressing various *Wnt* genes or *lacZ* were prepared essentially as described (Pear et al., 1993). Full-length cDNAs encoding *Wnt-1* (van Ooyen and Nusse, 1984), *Wnt-3a* (Roelink and Nusse, 1991), *Wnt-4*, *Wnt-5a*, *Wnt-7a*, *Wnt-7b* (Gavin et al., 1990), *Wnt-11* (Kispert et al., 1996) and *lacZ* were cloned into the retroviral expression vector pLNCX which confers expression of foreign genes under the control of the CMV promotor (Miller and Rosman, 1989). Bosc23 packaging cells were transfected with recombinant DNA constructs. Viral supernatants were collected 48-72 hours later and used to infect NIH3T3 cells. After 10 days of selection in G418, pools of cells were used for recombination experiments. 50,000 cells were plated in 50 μl of medium on polycarbonate filter and grown for 18-24 hours at 37°C in 5% CO_2.

Organ culture techniques

Metanephric kidneys from SW or *Wnt-4* intercrosses were dissected in PBS. Metanephric mesenchyme was dissected manually from the ureter (bud stage, 10.75 d p.c., to early T stage, 11.5 d p.c.), following a 2 minute incubation in 3% pancreatin/trypsin (Gibco-BRL) in Tyrode's solution. In recombination experiments with wild-type mesenchymes samples were pooled before being distributed to individual experiments. In experiments with *Wnt-4* mutant embryos metanephric mesenchyme from each kidney of the embryo was kept separate. The remainder of an embryo was used for genotyping by Southern analysis. In recombination experiments with dorsal spinal cord metanephric mesenchyme from two kidneys was surrounded by two dissected pieces of dorsal spinal cord from the same embryo on a 1 μm polycarbonate filter (Costar). For direct recombination experiments with *Wnt* expressing cells two mesenchymes were placed on top of modified NIH3T3 cells. For transfilter experiments 50,000 cells in 50 μl medium were seeded on a 1 μm filter 18-24 hours prior to the recombination. Cells were then covered with a 1 μm filter and two mesenchymes placed on this filter. Filters (4-6 mm in size) were supported by stainless steel grids on the surface of the culture medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, 1× penicillin/streptomycin). Medium was changed every 2 days. For glycosaminoglycan dependence of induction the medium was supplemented with 30 mM $NaClO_3$ after 0 hours, 24 hours and 48 hours, respectively. In experiments concerning pore size dependence of induction the pore size of the upper filter in the transfilter set-up was varied from 0.05 μm, 0.1 μm, 0.4 μm, 0.8 μm to 1 μm. The number of cultures performed are indicated in the text. For marker experiments at least 6 specimens were processed.

For in situ hybridisation analysis filters were submerged in cold methanol for 10 seconds and then fixed in 4% paraformaldehyde in PBS overnight prior to stepwise transfer into methanol and storage at −20°C. For histological analysis filters were fixed in Bouin's solution and stored in 70% ethanol at 4°C.

In situ hybridisation analysis

In situ hybridisation analyses on whole mount cultures were performed as described (Kispert et al., 1996). Full-length cDNAs for *WT-1* (Pritchard-Jones et al., 1990), *Pax-2* (Dressler et al., 1990), *Pax-8* (Plachov et al., 1990), *Wnt-4* (Gavin et al., 1990) and *E-cadherin* (Ringwald et al., 1987) were labeled with digoxigenin for whole mount detection.

Histological analysis

Samples were dehydrated, embedded in wax and sectioned at 5 μm. Sections were dewaxed, rehydrated and stained with haematoxylin and eosin.

Documentation

Brightfield images of cultures and marker stainings were taken with a binocular on Kodak 64T slide film. Histological sections were photographed on the same film on a Leitz Axiophot. Slides were scanned and figures composed in Adobe Photoshop 4.0.

RESULTS

Spinal cord mimics a mesenchymal signal for tubule induction

The identification of *Wnt-4* as a mesenchymal signal essential for tubule formation provides an excellent new tool for readdressing the role of spinal cord explants as heterologous inducers of kidney tubulogenesis. Clearly, if the spinal cord mimics a ureteric signal upstream of *Wnt-4*, this signal would not rescue the mesenchymal requirement for *Wnt-4* in tubulogenesis. To test this possibility, isolated metanephric mesenchyme from individual embryos derived from intercrosses between mice heterozygous for a likely null allele of *Wnt-4* were cultured on a polycarbonate filter in direct contact with dorsal spinal cord from the same embryo. In the absence of spinal cord, all mesenchyme cultures rapidly degenerated as expected. Surprisingly, when cultured in the presence of spinal cord, mesenchyme from *Wnt-4* mutant embryos developed as well as that of wild-type or heterozygous siblings (Table 1). After 48 hours induction was visible as bright round zones of condensing mesenchyme. After 96 hours the zones of condensing mesenchyme had undergone epithelialisation to form complex tubules. At 144 hours epithelial tubular structures and glomeruli indicated that full differentiation of induced tubules occurred in all recombinants (Fig. 1). Thus, the induction of tubulogenesis in *Wnt-4* mutant mesenchyme indicates that spinal cord signaling acts by either mimicking the action of Wnt-4 itself, or a factor downstream of *Wnt-4*. Further, although *Wnt-4* is expressed in the spinal cord (Parr et al., 1993), the observation that spinal cord from *Wnt-4* mutants is capable of induction indicates that *Wnt-4* expression in the spinal cord is not essential for this process. This leaves open the involvement of other *Wnts* expressed in this tissue.

Table 1. Induction of tubulogenesis in *Wnt-4/Wnt-4* mutant metanephric mesenchyme by dorsal spinal cord

		#Induced/#Total		
Exp. #	# Recombinants	+/+	*Wnt-4*/+	*Wnt-4*/*Wnt-4*
1	8	2/2	5/5	1/1
2	7	1/1	3/3	3/3
3	7	3/3	3/3	1/1
4	5	1/1	3/3	1/1
5	9	3/3	4/4	2/2
6	11	7/7	4/4	–
7	11	3/3	4/4	4/4
Total	58	20/20	26/26	12/12

Isolated metanephric mesenchyme was recombined with dorsal spinal cord from the same embryo on a nucleopore filter. Induction was monitored by bright field microscopy. Embryos of a total of seven litters were analysed in this way.

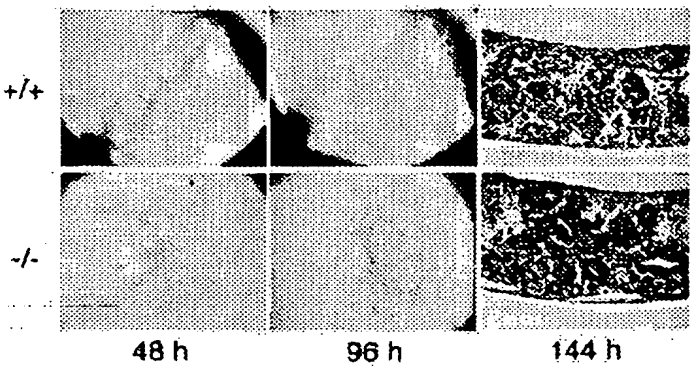


Fig. 1. Induction of tubulogenesis in wild-type and *Wnt-4* mutant metanephric mesenchyme by dorsal spinal cord. Isolated metanephric mesenchyme and dorsal spinal cord from the same 11.5 day embryo were recombined on a nucleopore filter. After 48 hours and 96 hours cultures were monitored as whole mounts using bright field microscopy, after 144 hours as histological sections. Induction of tubulogenesis in wild-type and *Wnt-4/Wnt-4* mutant metanephric mesenchyme were indistinguishable. After 48 hours induction was visible as bright round zones of condensing mesenchyme. After 96 hours zones of condensing mesenchyme had undergone epithelialisation to form complex tubules. At 144 hours epithelial tubular structures and glomeruli indicate a full differentiation of induced tubules in either case.

Various Wnts are sufficient to trigger tubulogenesis

In order to investigate whether *Wnt-4* is sufficient for tubulogenesis, and if this property is shared by other *Wnts* normally expressed in the spinal cord (Parr et al., 1993), we established NIH3T3 cell lines stably expressing various *Wnt* genes and performed direct recombinations between *Wnt* expressing cells and isolated wild-type metanephric mesenchyme. Co-cultures with *Wnt-1*, *Wnt-3a*, *Wnt-4*, *Wnt-7a* and *Wnt-7b* expressing cells developed on schedule with those induced by spinal cord, forming complex epithelial tubules with differentiated glomeruli at 144 hours (Fig. 2; Table 2). In contrast, cells expressing *Wnt-5a*, *Wnt-11* or a *lacZ* control did not support survival and differentiation of metanephric mesenchyme (Fig. 2; Table 2). Although we cannot exclude the possibility that the *Wnt-5a* and *Wnt-11* cell lines did not produce the respective Wnt protein, *Wnt* mRNA expression was comparable amongst the various lines.

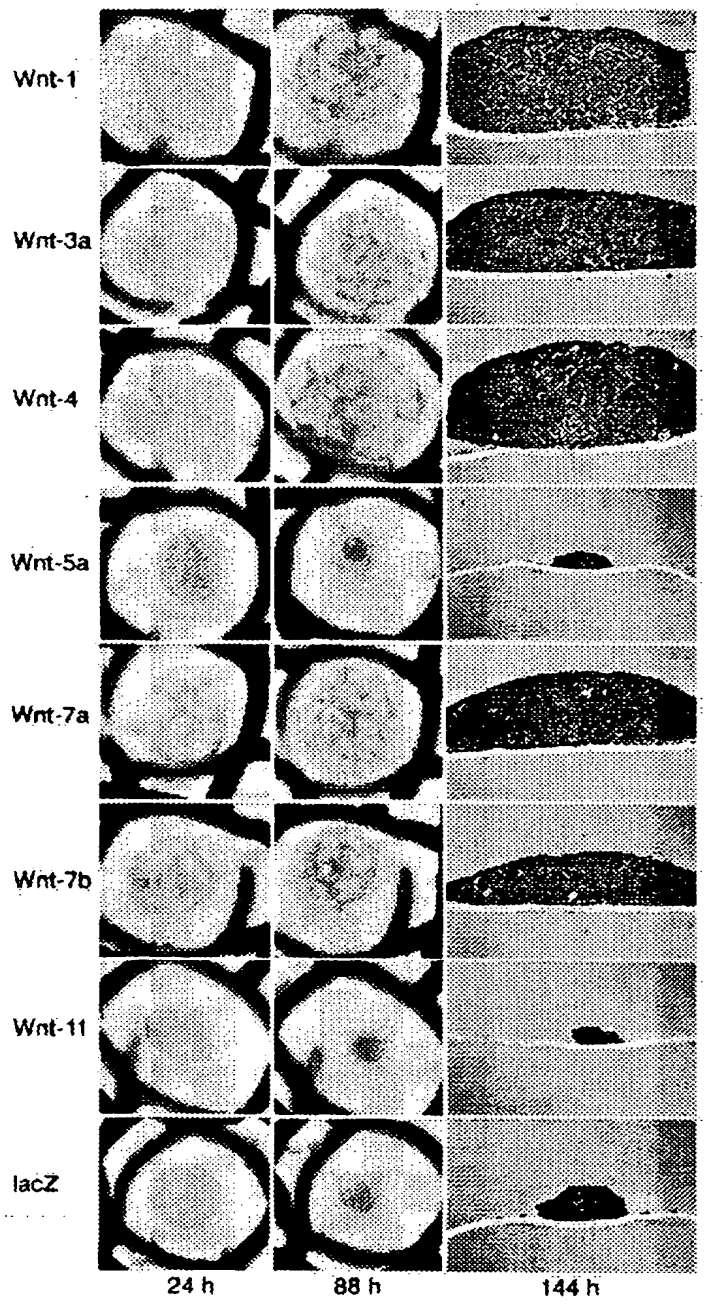


Fig. 2. Induction of tubulogenesis in isolated metanephric mesenchyme by NIH3T3 cells expressing various *Wnt* genes. Brightfield microscopy (24 hours, 88 hours) and histological analysis (144 hours) of direct recombinations between NIH3T3 cells expressing *Wnt* genes and isolated metanephric mesenchyme. After 24 hours bright zones indicating induction are visible in recombinants between wild-type mesenchyme and *Wnt-1*, *Wnt-3a*, *Wnt-4*, *Wnt-7a* and *Wnt-7b* expressing cells, respectively. These condensing mesenchymal cells have epithelialised and formed tubular structures after 88 hours. After 144 hours highly elaborate tubular structures are apparent. In contrast, cells expressing *Wnt-5a*, *Wnt-11*, or as a control *lacZ*, respectively, did not support survival and differentiation of metanephric mesenchyme.

Table 2. Induction of tubulogenesis in isolated metanephric mesenchyme by NIH3T3 cells expressing various *Wnt* genes

Cell line	#Induced/#Total
Wnt-1	16/16
Wnt-3a	14/14
Wnt-4	14/14
Wnt-5a	0/12
Wnt-7a	12/12
Wnt-7b	11/12
Wnt-11	0/12
lacZ	1/14
Mesenchyme	1/12

Isolated metanephric mesenchyme from 2-3 11.5 day kidneys was placed on top of NIH3T3 cells expressing various *Wnt* genes. As control mesenchymes were placed on NIH3T3 cells expressing *lacZ* and were placed onto filter without underlying cell layer, respectively. Induction was scored after 6 days using the morphological appearance of the culture (as documented by brightfield microscopy), and histological analysis of selected samples. For each cell type 2-3 independent experiments were performed.

These experiments suggest that a subset of *Wnt* genes, which includes *Wnt-4* and not *Wnt-11*, are able to induce tubule formation. As all of these are expressed in the spinal cord at the time of assay (Parr et al., 1993), it is likely that these signals account for the robust inducing activity of the spinal cord. However, of these *Wnt-4* is the only member which is actually expressed in and which is also required for mesenchymal aggregation (Stark et al., 1994).

Wnt-4 triggers the complete program of tubular differentiation

In order to investigate whether Wnt-4 is sufficient to induce fully developed tubules in isolated metanephric mesenchyme we analysed the induction properties of NIH3T3 cells expressing *Wnt-4* more carefully by assessing the differentiation state of the mesenchyme by histological and molecular criteria. Tubule induction by spinal cord was classically shown to work through polycarbonate filters of a certain pore size (Grobstein, 1956). We seeded *Wnt-4* expressing cells on one filter and separated these cells from isolated mesenchyme by another filter of 1 μm pore size. Induction took place transfilter (Fig. 3), though with a delay when compared with direct recombinants. Further, transfilter cultures where also less compact and flatter. Zones of condensed mesenchyme formed after 24 hours, aggregating mesenchyme and simple epithelial bodies after 48 hours, epithelial tubules after 96 hours and glomeruli by 8 days.

To verify that these morphological features reflected an underlying differentiation of the mesenchyme in response to Wnt-4 we examined the temporal and spatial expression of a number of molecular markers (Fig. 4). *WT-1* was broadly expressed after 1 day refining to small intensely labeled foci by 8 days of culture. This expression profile parallels the expression of this gene during metanephric development (Pritchard-Jones et al., 1990) which is first expressed in condensing mesenchyme, then in simple epithelial bodies before it is restricted to podocytes in the glomeruli. In the recombinants *WT-1* expression seems to mark glomeruli after 8 days in agreement with the histological analysis. Like *WT-1*, *Pax-2* is also broadly expressed after 1 day, but becomes restricted to epithelial bodies and is lost after 4 days reflecting

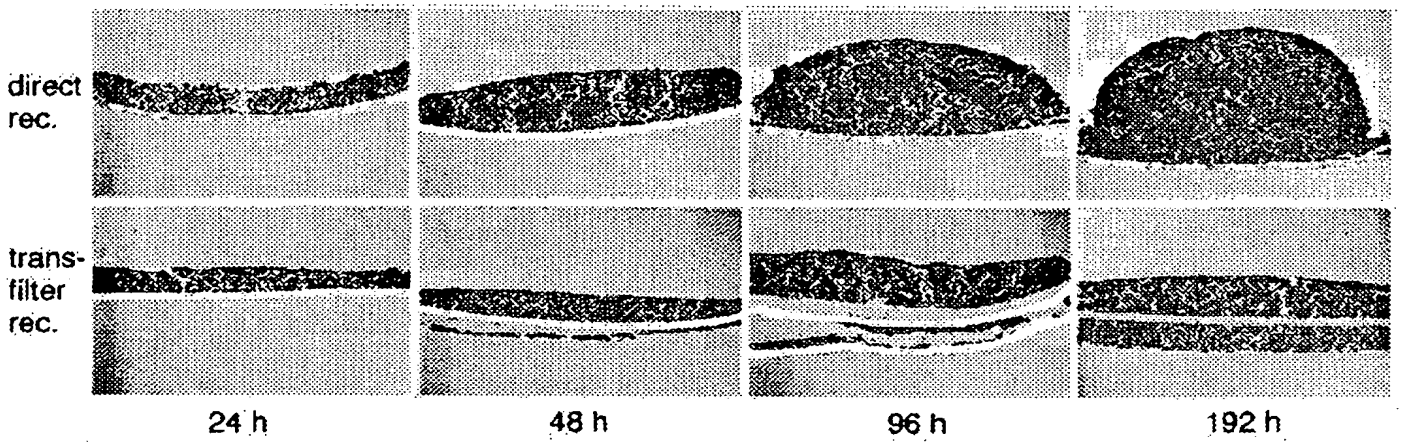


Fig. 3. Histological analysis of tubule induction in isolated metanephric mesenchyme by NIH3T3 cells expressing *Wnt-4*. NIH3T3 cells expressing *Wnt-4* were recombined with isolated metanephric mesenchyme directly (direct rec.) and in a transfilter set-up (transfilter rec.), respectively, and analysed by sectioning and histological staining after 24 hours, 48 hours, 96 hours and 192 hours of culture, respectively. Tubule induction in transfilter assays appeared slightly delayed compared to direct recombinations. After 48 hours zones of condensed and aggregated mesenchyme, after 96 hours epithelial tubules were apparent. After 8 days in culture fully differentiated tubular structures including glomeruli were noticed.

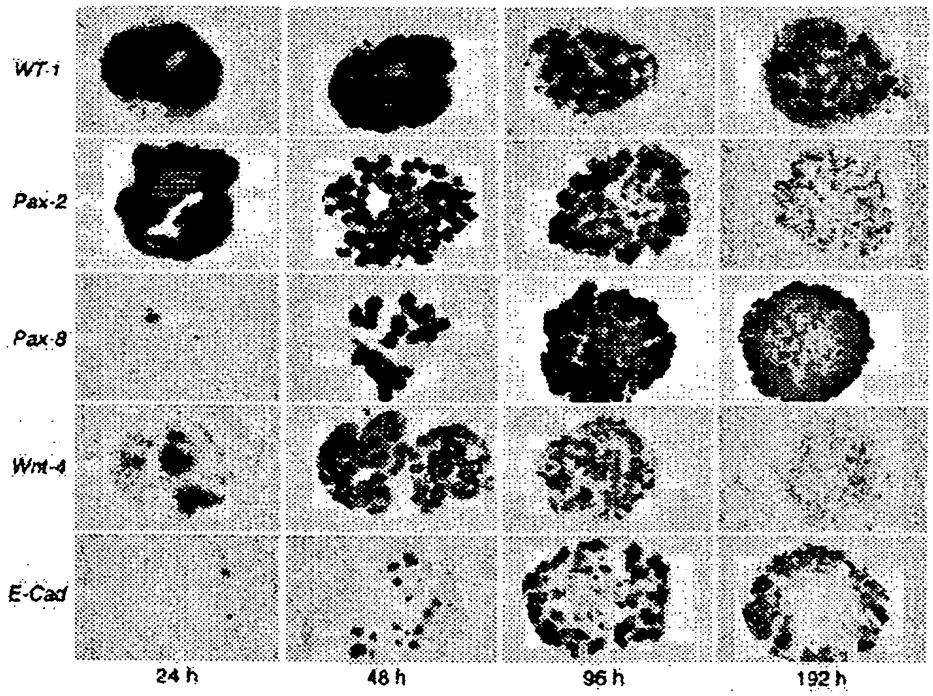


Fig. 4. Marker analysis of tubule induction in isolated metanephric mesenchyme by NIH3T3 cells expressing *Wnt-4*. NIH3T3 cells expressing *Wnt-4* were recombined with isolated metanephric mesenchyme in a transfilter set-up and scored for marker expression by in situ analysis after 24 hours, 48 hours, 96 hours and 192 hours of culture, respectively. Expression of *WT-1*, *Pax-2*, *Pax-8*, *Wnt-4* and *E-cadherin*, respectively, were in accordance with expression data known from in vivo and in vitro studies of tubular differentiation. See text for details.

initial expression in condensing metanephric mesenchyme, continuing expression in simple epithelial bodies and subsequent down-regulation as glomeruli start to differentiate (Dressler et al., 1990). *Wnt-4* is expressed in aggregating mesenchyme, in the epithelial bodies which they generate and is subsequently down-regulated as these mature into S-shaped bodies (Stark et al., 1994). *Pax-8*, a paired-box transcription factor, has a similar early expression to *Wnt-4* which has been shown to depend on Wnt-4 activity (Plachov et al., 1990; Stark et al., 1994). In cultures, *Wnt-4* is transiently expressed between 24 hours and 96 hours, peaking at 48 hours. *Pax-8* expression extends longer in S-shaped bodies. *E-cadherin* which is expressed in the proximal tubules in vivo (Vestweber et al., 1985) is present after 24 hours and is maintained consistent with the differentiation of epithelial tubules along the proximal distal axis. Thus, both the molecular and morphological analysis indicate that tubulogenesis in isolated metanephric mesenchyme induced by Wnt-4 follows a similar progression to that observed in the metanephric kidney in vivo. At the stage at which we isolate the metanephric mesenchyme

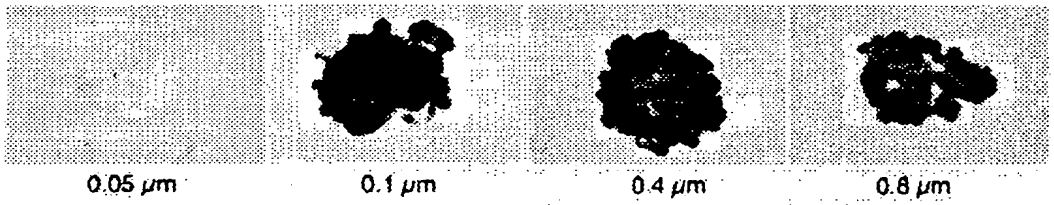


Fig. 5. Pore size dependence of tubule induction by *Wnt-4* expressing cells. NIH3T3 cells expressing *Wnt-4* were recombined with isolated metanephric mesenchyme in a transfilter set-up with various pore sizes of the nucleopore filter. Induction was scored after 4 days by *Pax-8* expression in whole mount in situ analysis. Pore sizes of 0.1 μm and bigger supported full induction of metanephric mesenchyme whereas 0.05 μm pore size dramatically reduced or in most cases abolished induction.

(T-stage of the ureter) initial ureteric signaling has occurred as evidenced by the condensation of mesenchyme around the tip of the ureteric bud. However, this alone is insufficient to support mesenchymal survival and tubulogenesis. In contrast, *Wnt-4* expressing cells are sufficient to support these processes. In order to exclude the possibility that Wnt-4 only maintains *Wnt-4* expression in the isolated mesenchyme we also used mesenchyme derived from 10.75 d p.c. embryos when the ureter bud had just emerged and the metanephric mesenchyme can first be identified. *Wnt-4* expressing cells triggered the complete differentiation program as judged by brightfield observation (12 out of 12 cases) and by molecular criteria (*Pax-8* induction in 8 out of 8 cases after 4 days of culture).

Wnt-4 signaling requires cell contact

We explored tubule induction in isolated metanephric mesenchyme with respect to filter pore size. Experiments using the spinal cord as a heterologous inducer suggest a requirement for cell-cell contact as pore sizes below 0.1 μm, which prevent the extension of cytoplasmic processes, block induction (Saxen, 1987). Transfilter cultures with *Wnt-4* expressing NIH3T3 cells were performed with separating filters ranging from 0.05 μm to 1 μm in pore size, scoring for *Pax-8* induction after 4 days of culture. Pore sizes of 0.1 μm and above supported induction whereas pores of 0.05 μm almost completely abolished induction leading to degeneration of the mesenchyme (Fig. 5; Table 3). Further, we were not able to induce tubulogenesis with supernatants from *Wnt-4* expressing cells (data not shown). Thus, Wnt-4 may act as an insoluble cell bound factor. Such a mode of action agrees well with the known tight association of Wnt proteins with ECM (Bradley and Brown, 1990; Papkoff and Schryver, 1990; Burrus and McMahon, 1995). It also makes it unlikely that Wnt-4 mediated induction occurs through a secondary, soluble factor.

Table 3. Induction of tubulogenesis in isolated metanephric mesenchyme by NIH3T3 cells expressing *Wnt-4* in transfilter assays with increasing pore size

Pore size (μm)	#Induced/#Total
0.05	3*/13
0.1	14/16
0.4	14/14
0.8	6/6
1	3/3

Isolated metanephric mesenchyme was placed on top of NIH3T3 cells expressing *Wnt-4* in a transfilter set-up. Induction was scored after 4 days with in situ hybridisation analysis using *Pax-8* as a probe.

*In each of the specimens scored as induced only 1-4 spots of *Pax-8* expression were seen in contrast to 15-30 with all the other pore sizes.

Wnt-4 signaling requires sulphated glycosaminoglycans

Wnt signaling has been reported to depend on sulfated glycosaminoglycans (GAG)s which might act as cofactors for binding the Wnt protein on the responsive cell (Kispert et al., 1996; Hacker et al., 1997). We were therefore interested to see whether the presence of 30 mM $NaClO_3$, which is known to be a competitive inhibitor of sulfation of GAGs (Kjellen and Lindahl, 1991), may influence induction in our assay. We added this compound at the start of transfilter culture, or 24 and 48 hours later. As a control chlorate was omitted completely. *Pax-8* expression was again scored as a marker for tubule induction after 4 days of culture (Table 4; Fig. 6). When chlorate was added at 0 hours mesenchyme degenerated and *Pax-8* expression was consequently negative. However, addition of chlorate after 24 hours did not influence *Pax-8* expression. Hence, GAGs do not seem to be involved in tubule maturation and differentiation, in agreement with other reports (Davies et al., 1995). Tubule induction does, however, depend on sulfated GAGs in the first 24 hours, the period essential for complete induction by the spinal cord. Although our results point to an important role for GAGs in Wnt-4's action, their precise role remains unclear.

Wnt-4 signaling is only required to trigger tubulogenesis

In order to test whether *Wnt-4* expressing cells can rescue a

Table 4. Induction of tubulogenesis in isolated metanephric mesenchyme by NIH3T3 cells expressing *Wnt-4* in the presence of 30 mM $NaClO_3$

30 mM $NaClO_3$ added after time in culture (hours)	#Induced/#Total
0	0/19
24	12/19
48	14/17
-	12/15

Isolated metanephric mesenchyme was placed on top of NIH3T3 cells expressing *Wnt-4* in a transfilter set-up. 30 mM $NaClO_3$ was added to the medium after the indicated times of setting-up the culture. Induction was scored after 4 days with in situ hybridisation analysis using *Pax-8* as a probe.

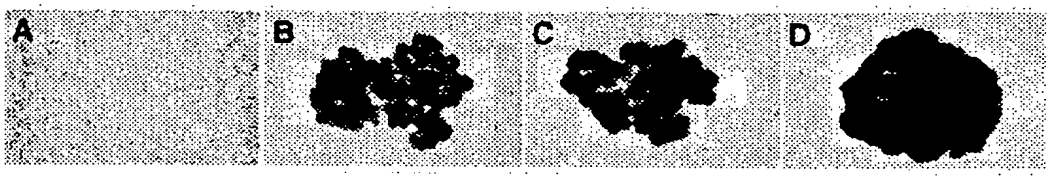


Fig. 6. Glycosaminoglycan dependence of tubule induction by *Wnt-4* expressing cells. NIH3T3 cells expressing *Wnt-4* were recombined with isolated metanephric mesenchyme in a transfilter set-up with addition of 30 mM $NaClO_3$ in the medium. Induction was scored after 4 days by *Pax-8* expression using whole mount in situ hybridisation analysis. Addition of 30 mM $NaClO_3$ after 24 hours (B) or 48 hours (C) of culture did not affect tubule induction compared to untreated controls (D) whereas administration of 30 mM $NaClO_3$ at the beginning of the culture (A) abrogated tubule induction completely.

Wnt-4 mutant mesenchyme we performed direct recombination experiments in culture. Interestingly, *Wnt-4* expressing cells were equally efficient at inducing tubule formation in wild type or *Wnt-4* mutant metanephric mesenchyme (Table 5). Brightfield microscopy and histological analysis of specimen after 6 days in culture revealed the full spectrum of tubular differentiation including glomerulus formation (Fig. 7). Thus, as with spinal cord mediated induction, *Wnt-4* expression in the mesenchyme itself is not required for tubule formation, but supplying *Wnt-4* in adjacent cells is sufficient to trigger the inductive process. These results suggest that whereas *Wnt-4* plays an essential role in initial tubulogenesis, it may not be required for later morphogenesis of the tubule. In agreement with the fact that various *Wnt* genes can trigger tubulogenesis in wild-type mesenchyme *Wnt-1* expressing cells were also sufficient to trigger tubulogenesis in mesenchyme mutant for *Wnt-4* (Table 5).

Table 5. Induction of tubulogenesis in *Wnt-4/Wnt-4* mutant metanephric mesenchyme by NIH3T3 cells expressing *Wnt-4* or *Wnt-1*

		#Induced/#Total		
Exp. #	# Recombinants	*+/+*	*Wnt-4/+*	*Wnt-4/Wnt-4*
With NIH3T3 cells expressing *Wnt-4*:				
4	42	7/7	18/18	17/17
With NIH3T3 cells expressing *Wnt-1*:				
2	20	5/5	11/12	3/3

Isolated metanephric mesenchyme from embryos of *Wnt-4/+* intercrosses was placed on top of NIH3T3 cells expressing *Wnt-4* or *Wnt-1*. Induction was scored after 6 days using the morphological appearance of the culture (as documented by brightfield microscopy).

DISCUSSION

Mammalian metanephric development is a highly coordinated process characterised by a continuous interaction of the epithelial ureter and the surrounding metanephric mesenchyme. Classical organ culture experiments have pointed to the fact that these two compartments achieve coordinated development by use of reciprocal signaling systems. First, the metanephric blastema induces a bud from the adjacent nephric duct which invades and branches into the mesenchyme. This process appears to be mediated by GDNF which is secreted by the metanephric mesenchyme and sensed by the c-ret/GDNFRa receptor complex on the ureter (see Sariola and Sainio, 1997, for review). Next, the metanephric mesenchyme undergoes tubulogenesis upon a permissive stimulus from the ureter.

Since ureter itself is a weak inducer, indeed, ureter has never been proven to induce in transfilter assays of tubule induction, heterologous tissues, most notably dorsal spinal cord, have been used for a long time to mimic ureteric signaling. More recently, several studies have implicated *Wnt* genes in ureteric signaling and in dorsal spinal cord activity. The four key observations are:

(1) *Wnt-11* is expressed in the ureter tips (Kispert et al., 1996).

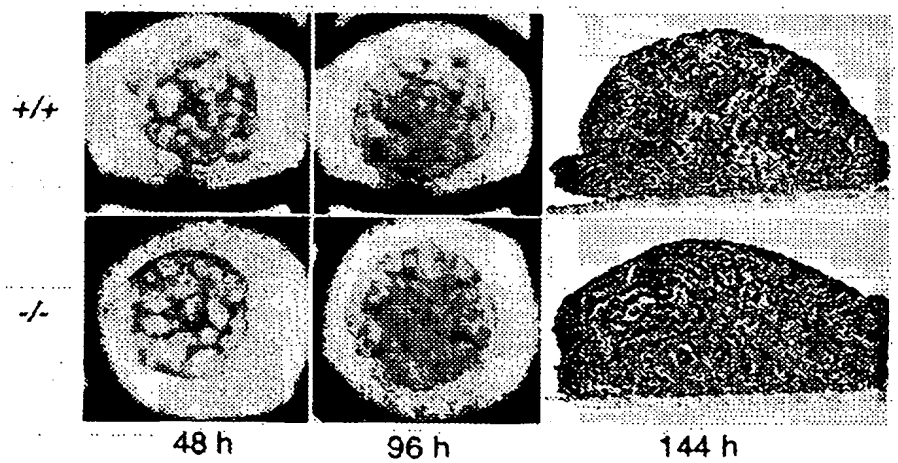


Fig. 7. Induction of tubulogenesis in wild-type and *Wnt-4* mutant metanephric mesenchyme by NIH3T3 cells stably expressing *Wnt-4*. Isolated metanephric mesenchyme was placed on top of NIH3T3 cells expressing *Wnt-4* which were supported by a nucleopore filter. After 48 hours and 96 hours cultures were monitored as whole mounts using bright field microscopy, after 144 hours as histological sections. Induction of tubulogenesis in wild-type and *Wnt-4/Wnt-4* mutant metanephric mesenchyme by *Wnt-4* expressing cells were indistinguishable.

(2) Cells expressing *Wnt-1* can induce tubulogenesis (Herzlinger et al., 1994).

(3) *Wnt-4* is a critically required for epithelialisation of condensed mesenchyme (Stark et al., 1994).

(4) Various *Wnt* genes are expressed in the spinal cord (Parr et al., 1993).

With respect to these findings our experimental data are of dual importance. First, they point to the nature of signals emitted by the heterologous inducer spinal cord and clarify the action of these signals with respect to ureter signaling. Second, they argue for a decisive role for Wnt-4 signaling in the mesenchyme, adding another signaling system to tubulogenesis, a process which has not been fully appreciated up to now.

Spinal cord signaling and Wnt-4

Classical kidney organ culture experiments have identified numerous tissues of diverse embryonic origins, which can elicit tubulogenesis in isolated metanephric mesenchyme (Unsworth and Grobstein, 1970; Saxen, 1987). As these tissues are robust inducers, they, rather than the ureter itself, have been widely used to define the process of kidney tubule induction. In our recombination experiments we have demonstrated that these inducers, exemplified by dorsal spinal cord tissue, can trigger tubulogenesis in *Wnt-4* mutant mesenchyme. Therefore, it seems unlikely that the signals emitted from the spinal cord, and possibly other heterologous inducing tissues, mimic an endogenous ureteric signal(s) which would still require *Wnt-4* for tubule formation. We suggest they act on the level of *Wnt-4* in the mesenchyme. Since dorsal spinal cord used in the assays was also mutant for *Wnt-4* other *Wnts* may replace Wnt-4 activity in the mesenchyme. By using cell lines expressing various *Wnt* genes we show that *Wnt-1*, *Wnt-3a*, *Wnt-7a*, *Wnt-7b*, all of which are expressed in spinal cord, can evoke tubulogenesis in isolated metanephric mesenchyme. The additive action of several Wnt proteins may also explain why dorsal spinal cord represents such a strong source of inducer. Further, *Wnts* are widely expressed during embryogenesis (Parr et al., 1993; Lee et al., 1995) and could explain why so many tissues can trigger tubulogenesis in isolated metanephric mesenchyme.

In summary, our results suggest a different interpretation of the use of kidney cultures to elucidate the nature of the ureteric signal involved in inducing the mesenchyme. Experiments which have used heterologous sources of tubule inducers, most notably the spinal cord, may not have been investigating the nature of ureteric signaling, but rather the mesenchymal action of signals such as Wnt-4. At present, the exact nature of ureteric signaling remains obscure. It seems clear that a primary signal from the ureter leads to survival and initial condensation of metanephric mesenchyme. This primary signal might be required for a sufficient length of time to allow auto-induction of the mesenchyme by Wnt-4. Alternatively, a secondary signal from the ureter tip might be necessary to induce *Wnt-4* expression in aggregating mesenchyme.

Our experiments argue against a role for Wnt-11 as a ureteric signal for mesenchymal aggregation. As a secreted glycoprotein expressed at the ureter tip Wnt-11 was a prime candidate for such an activity. However, we were not able to get tubulogenesis with cells expressing *Wnt-11*. Recent loss-of-function experiments also support this notion. Mice homozygous for a likely null mutation of *Wnt-11* do not exhibit an overt kidney phenotype (S. Vainio, A. Kispert and A. P. McMahon, unpublished observation). At this time, the function of *Wnt-11* is unclear.

Wnt-4 is a mesenchymal signal for tubulogenesis

Analysis of *Wnt-4* mutants has demonstrated a critical role for *Wnt-4* in kidney development. Homozygous pups die 24 hours after birth due to small agenic kidneys consisting of undifferentiated mesenchyme intermingled with collecting duct tissue. Histological and marker analysis revealed that primary condensation of mesenchymal cells around the ureter tips as well as ureteric branching occurs normally. However, mutant kidneys quickly become growth retarded and the mesenchyme remains undifferentiated lacking pretubular cell aggregates and epithelial tubules. Since kidney size as well as cell death initially remain unaffected, proliferation is unlikely to be controlled by *Wnt-4*. Rather, the lack of *Wnt-4* expression itself and of epithelial structures in the mutant mesenchyme argues that Wnt-4 may autoinduce the epithelialisation of condensed mesenchyme. In this study we have shown that mesenchymally derived *Wnt-4* is not only required but also sufficient for induction of tubulogenesis in the mammalian kidney. Judging by histological and molecular markers Wnt-4 can elicit the complete program of tubular differentiation in isolated metanephric mesenchyme. The activity of Wnt-4 contrasts with other factors thought to regulate mesenchymal development. For example, FGF (Perantoni et al., 1995) and EGF (Weller et al., 1991; Koseki et al., 1992) can both support mesenchymal survival but are not sufficient for tubulogenesis. Like Wnt-4, BMP-7 has been suggested to induce tubules (Vukicevic et al., 1996) but loss-of-function studies indicate it is not essential for tubule formation in vivo as some glomeruli form in *BMP7* mutants (Dudley et al., 1995; Luo et al., 1995). In contrast, loss of *Wnt-4* leads to a complete absence of glomeruli.

Wnt-4 activity shows all the characteristics which have previously been ascribed to induction by dorsal spinal cord tissue. Signaling is cell-contact dependent. Below a certain pore size in the transfilter assay the formation of cellular processes which penetrate the filter pores is inhibited and isolated mesenchyme degenerates. Cell contact dependence agrees well with the fact that Wnt proteins interact with extracellular matrix (ECM) components (Bradley and Brown, 1990; Papkoff and Schryver, 1990; Burrus, 1994; Burrus and McMahon, 1995). The chlorate inhibition experiments defines a critical period of 24 hours for induction. This is in agreement with classical studies which showed that the inducer tissue can be removed after this time with tubulogenesis proceeding undisturbed (Saxen, 1987). Possibly, every cell has to get in contact with the inducer to initiate tubulogenesis. We suggest that further differentiation, i.e. aggregation and epithelialisation of mesenchymal cells is only initiated when a certain number of cells (a small community) has received the Wnt-4 signal. At this time mesenchymal development is independent of ureteric signaling.

Chlorate acts as a competitive inhibitor of sulphotransferases and inhibits the sulphation of glycosaminoglycans (Kjellen and Lindahl, 1991). Our inhibition studies point to a critical role of these ECM compounds in tubulogenesis. Numerous studies have shown that branching morphogenesis of the ureter as well

as branching of other epithelia requires an intact ECM (Platt et al., 1987; Davies et al., 1995; Roskelly et al., 1995; Kispert et al., 1996). Since presence of chlorate after 24 hours does not influence tubulogenesis GAGs do not seem to be involved in tubule maturation and differentiation, in agreement with other reports (Davies et al., 1995). Tubule induction does, however, depend on sulfated GAGs in the first 24 hours, the period essential for complete induction by the spinal cord. Although our results point to an important role for GAGs in Wnt-4's action, their precise role remains unclear. It is possible that chlorate acts on the Wnt secreting cell. Recent experiments in the fly which indicate that GAG synthesis is also required for *wingless* (*Drosophila Wnt-1*) signaling (Reichsman et al., 1996; Binari et al., 1997; Hacker et al., 1997; Haerry et al., 1997) suggest that GAGs are required in the responsive cell for signal transduction. Most likely as is the case for FGFs, GAGs may act as co-receptors, facilitating presentation or increasing the local concentration of the ligand.

Wnt-4 expression in the metanephric mesenchyme is initiated in the aggregating mesenchyme and maintained in the comma shaped bodies before it is downregulated in S-shaped bodies. Therefore, *Wnt-4* might have a later function in tubulogenesis which is masked in the earlier requirement to form a tubule. However, cells expressing *Wnt-4* are sufficient to induce tubulogenesis in *Wnt-4* mutant mesenchyme arguing that *Wnt-4* expression may not have a later role. It seems that Wnt-4 probably acts as a trigger to start an intrinsic program in the mesenchymal cells which then proceed to form complex nephron like structures. From our experiments it is not clear whether in vivo Wnt-4 acts in an autocrine or a paracrine fashion. However, it seems clear that the signal acts very locally. Interestingly, although these experiments demonstrate that *Wnt-4* is itself a target of its own signaling activity, induced metanephric mesenchyme is not able to induce tubule formation when recombined with uninduced mesenchyme. This lack of homeogenetic induction could be taken as evidence against Wnt-4 acting as a mesenchymally derived tubule inducer. However, there are many other explanations for this result. For example lateral inhibition mechanism as is commonly used in fine grained patterning may limit Wnt-4 action. Alternatively, levels of induced Wnt-4 may not be high enough to induce more mesenchymal cells. More directly, a number of potential Wnt antagonists have been described that could potentially modulate Wnt-4 signaling (Moon et al., 1997; Rattner et al., 1997; Glinka et al., 1998).

Our view is that kidney tubulogenesis is a multi-step process with a hierarchy of signaling systems. A permissive signal from the ureter to the mesenchyme triggers survival and tubulogenesis in the mesenchyme, signals from the mesenchyme to the ureter are required for proliferation and branching morphogenesis of the ureter. Most likely, other signaling systems within the ureter are required for local adhesion and proliferation changes which may mediate branching morphogenesis, and within the mesenchyme for tubulogenesis as evidenced by the role of *Wnt-4*. Taking the complexity of developmental events in the mesenchyme into account, it is conceivable that additional signaling systems control the ratio between interstitial and metanephrogenic cells, between condensing and non-condensing cells, and the maintenance of the mesenchymal stem cells in the periphery. Undoubtedly, the functional analysis of other secreted factors will shed light on these diverse events in kidney development.

We thank Bénédicte Haenig for excellent technical assistance, and Scott Lee and Michael Leitges for critically reading the manuscript. We are grateful to Peter Gruss for the Pax-8 cDNA, Greg Dressler for the Pax-2 cDNA, Rolf Kemler for the E-Cadherin cDNA, Nick Hastie for the WT-1 cDNA, Jan Kitajewski for providing the pLNCX and the pLNCX.Wnt-1 plasmids, and Warren Pear for the Bosc23 packaging cell line. Work in A.P.M's laboratory was supported by grants from the National Institute of Health. A.K. was supported by the Max-Planck-Society and a long term postdoctoral fellowship by the Human Science Frontier Organisation. A.K. thanks Rolf Kemler for support. S.V. was supported by the Academy of Finland, the Alfred Kordelin Foundation, the European Molecular Biology Organization and the Sigrid Jusélius Foundation.

REFERENCES

Binari, R. C., Staveley, B. E., Johnson, W. A., Godavarti, R., Sasisekharan, R. and Manoukian, A. S. (1997). Genetic evidence that heparin-like glycosaminoglycans are involved in wingless signaling. *Development* **124**, 2623-2632.

Bradley, R. S. and Brown, A. M. C. (1990). The proto-oncogene int-1 encodes a secreted protein associated with the extracellular matrix. *EMBO J.* **9**, 1569-1575.

Burrus, L. W. (1994). Wnt-1 as a short-range signaling molecule. *BioEssays* **16**, 155-157.

Burrus, L. W. and McMahon, A. P. (1995). Biochemical analysis of murine Wnt proteins reveals both shared and distinct properties. *Exp. Cell Res.* **220**, 363-373.

Davies, J., Lyon, M., Gallagher, J. and Garrod, D. (1995). Sulphated proteoglycan is required for collecting duct growth and branching but not nephron formation during kidney development. *Development* **121**, 1507-1517.

Dressler, G. Y., Deutsch, U., Chowdhury, K., Nornes, H. O. and Gruss, P. (1990). *Pax2*, a new murine paired-box-containing gene and its expression in the developing excretory system. *Development* **109**, 787-795.

Dudley, A. T., Lyons, K. M. and Robertson, E. J. (1995). A requirement for bone morphogenetic protein-7 during development of the mammalian kidney and eye. *Genes Dev.* **9**, 2795-2807.

Gavin, B. J., McMahon, J. A. and McMahon, A. P. (1990). Expression of multiple novel *Wnt-1/int-1*-related genes during fetal and adult mouse development. *Genes Dev.* **4**, 2319-2332.

Glinka, A., Wu, W., Delius, H., Monaghan, A. P., Blumenstock, C. and Niehrs, C. (1998). Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. *Nature* **391**, 357-362.

Grobstein, C. (1953). Inductive epithelio-mesenchymal interactions in cultured organ rudiments of the mouse. *Science* **118**, 52-55.

Grobstein, C. (1956). Transfilter induction of tubules in mouse metanephrogenic mesenchyme. *Exp. Cell Res.* **10**, 424-440.

Hacker, U., Lin, X. and Perrimon, N. (1997). The Drosophila sugarless gene modulates Wingless signaling and encodes an enzyme involved in polysaccharide biosynthesis. *Development* **124**, 3565-3573.

Haerry, T. E., Heslip, T. R., Marsh, J. L. and O'Connor, M. B. (1997). Defects in glucuronate biosynthesis disrupt Wingless signaling in Drosophila. *Development* **124**, 3055-3064.

Hammerman, M. R. (1995). Growth factors in renal development. *Semin. Nephrol.* **15**, 291-299.

Herzlinger, D., Qiao, J., Cohen, D., Ramakrishna, N. and Brown, A. M. C. (1994). Induction of kidney epithelial morphogenesis by cells expressing *Wnt-1*. *Dev. Biol.* **166**, 815-818.

Kispert, A., Vainio, S., Shen, L., Rowitch, D. R. and McMahon, A. P. (1996). Proteoglycans are required for maintenance of *Wnt-11* expression in the ureter tips. *Development* **122**, 3627-3637.

Kjellen, L. and Lindahl, U. (1991). Proteoglycans: structure and functions. *Annu. Rev. Biochem.* **60**, 443-475.

Koseki, C., Herzlinger, D. and al-Awqati, Q. (1992). Apoptosis in metanephric development. *J. Cell Biol.* **119**, 1327-1333.

Lechner, M. A. and Dressler, G. R. (1997). The molecular basis of embryonic kidney development. *Mech. Dev.* **62**, 105-120.

Lee, S. M. K., Dickinson, M. E., Parr, B. A., Vainio, S. and McMahon, A. P. (1995). Molecular genetic analysis of Wnt signals in mouse development. *Semin. Dev. Biol.* **6**, 267-274.

Luo, G., Hofman, C., Bronckers, A. L. L. J., Sohocki, M., Bradley, A. and Karsenty, G. (1995). BMP-7 is an inducer of nephrogenesis, and is required for eye development and skeletal patterning. *Genes Dev.* **9**, 2808-2820.

Miller, A. D. and Rosman, G. J. (1989). Improved retroviral vectors for gene transfer and expression. *Biotechniques* **7**, 980-990.

Moon, R. T., Brown, J. D., Yang-Snyder, J. A. and Miller, J. R. (1997). Structurally related receptors and antagonists compete for secreted Wnt ligands. *Cell* **88**, 725-728.

Papkoff, J. and Schryver, B. (1990). Secreted *int-1* protein is associated with the cell surface. *Mol. Cell. Biol.* **10**, 2723-2730.

Parr, B. A., Shea, M. J., Vassileva, G. and McMahon, A. P. (1993). Mouse *Wnt* genes exhibit discrete domains of expression in the early embryonic CNS and limb buds. *Development* **119**, 247-261.

Pear, W. S., Nolan, G. P., Scott, M. L. and Baltimore, D. (1993). Production of high-titer helper-free retroviruses by transient transfection. *Proc. Nat. Acad. Sci. USA* **90**, 8392-8396.

Perantoni, A. O. (1991). Induction of tubules in rat metanephrogenic mesenchyme in the absence of an inductive tissue. *Differentiation* **48**, 25-31.

Perantoni, A. O., Dove, L. F. and Karavanova, I. (1995). Basic fibroblast growth factor can mediate the early inductive events in renal development. *Proc. Nat. Acad. Sci. USA* **92**, 4696-4700.

Plachov, D., Chowdhury, K., Walther, C., Simon, D., Guenet, J.-L. and Gruss, P. (1990). Pax8, a murine paired box gene expressed in the developing excretory system and the thyroid gland. *Development* **110**, 643-651.

Platt, J. L., Brown, D. M., Granlund, K., Oegema, T. R. j. and Klein, D. J. (1987). Proteoglycan metabolism associated with mouse metanephric development: morphologic and biochemical effects of b-D-xyloside. *Dev. Biol.* **123**, 293-306.

Pritchard-Jones, K., Fleming, S., Davidson, D., Bickmore, W., Porteous, D., Gosden, C., Bard, J., Buckler, A., Pelletier, J., Housman, D. E., van Heyningen, V. and Hastie, N. (1990). The candidate Wilms' tumour gene is involved in genitourinary development. *Nature* **346**, 194-197.

Rattner, A., Hsieh, J. C., Smallwood, P. M., Gilbert, D. J., Copeland, N. G., Jenkins, N. A. and Nathans, J. (1997). A family of secreted proteins contains homology to the cysteine-rich ligand-binding domain of frizzled receptors. *Proc. Nat. Acad. Sci. USA* **94**, 2859-2863.

Reichsman, F., Smith, L. and Cumberledge, S. (1996). Glycosaminoglycans can modulate extracellular localization of the wingless protein and promote signal transduction. *J. Cell Biol.* **135**, 819-27.

Ringwald, M., Schuh, R., Vestweber, D., Eistetter, H., Lottspeich, F., Engel, J., Dolz, R., Jahnig, F., Epplen, J., Mayer, S. et al. (1987). The structure of cell adhesion molecule uvomorulin. Insights into the molecular mechanism of Ca^{2+}-dependent cell adhesion. *EMBO J.* **6**, 3647-3653.

Roelink, H. and Nusse, R. (1991). Expression of two members of *Wnt* gene family during mouse development - restricted temporal and spatial patterns in the developing neural tube. *Genes Dev.* **5**, 381-388.

Roskelly, C. D., Srebrow, A. and Bissell, M. J. (1995). A hierarchy of ECM-mediated signalling regulates tissue-specific gene expression. *Curr. Opin. Cell Biol.* **7**, 736-747.

Sainio, K., Suvanto, P., Davies, J., Wartiovaara, J., Wartiovara, K., Saarma, M., Ammae, U., Meng, X., Lindahl, M., Pachnis, V. and Sariola, H. (1997). Glial-cell-line-derived neurotrophic factor is required for bud initiation from ureteric epithelium. *Development* **124**, 4077-4087.

Sariola, H. and Sainio, K. (1997). The tip-top branching ureter. *Curr. Opin. Cell Biol.* **9**, 877-884.

Saxen, L. (1987). Organogenesis of the kidney. Cambridge, UK, Cambridge University Press.

Stark, K., Vainio, S., Vassileva, G. and McMahon, A. P. (1994). Epithelial transformation of metanephric mesenchyme in the developing kidney regulated by *Wnt-4*. *Nature* **372**, 679-683.

Unsworth, B. and Grobstein, C. (1970). Induction of kidney tubules in mouse metanephric mesenchyme by various embryonic mesenchymal tissues. *Dev. Biol.* **21**, 547-556.

Vainio, S. and Muller, U. (1997). Inductive tissue interactions, cell signaling, and the control of kidney organogenesis. *Cell* **90**, 975-978.

van Ooyen, A. and Nusse, R. (1984). Structure and nucleotide sequence of the putative mammary oncogene int-1; proviral insertions leave the protein-encoding domain intact. *Cell* **39**, 233-240.

Vestweber, D., Kemler, R. and Ekblom, P. (1985). Cell-adhesion molecule uvomorulin during kidney development. *Dev. Biol.* **112**, 213-221.

Vukicevic, S., Kopp, J. B., Luyten, F. P. and Sampath, T. K. (1996). Induction of nephrogenic mesenchyme by osteogenic protein 1 (bone morphogenetic protein 7). *Proc. Nat. Acad. Sci. USA* **93**, 9021-9026.

Weller, A., Sorodin, L., Illgen, E.-M. and Ekblom, P. (1991). Development and growth of mouse embryonic kidney in organ culture and modulation of developmental by soluble growth factor. *Dev. Biol.* **144**, 248-261.